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Oligomers of beta-L-arabinosides of hydroxyproline : synthesis of the carbohydrate epitope of the Art v 1 allergen

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OLIGOMERS OF BETA-L-ARABINOSIDES OF HYDROXYPROLINE: SYNTHESIS OF THE CARBOHYDRATE EPITOPE OF THE ART V 1 ALLERGEN

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To My Family…

To my wife Thao, without whom I would not be the man I am today.

To my son Andrew, whose mere existence inspires me to become an even better man.

To my mom and dad, I love you more than you'll ever know, more than I'll ever say…

…This Dissertation Is For You

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LIST OF ABBREVIATIONS AND SYMBOLS

Å	Angstrom
Ac	Acetyl
AgOTf	silver triflate
AGP	arabinogalactan proteins or arabinogalactan polysaccharides
Amb	*Ambrosia artemisiifolia*
Ara(*f*)	arabinose(furanose)
Art v 1	*Artemisia vulgaris*
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazol-1-yloxytris(dimethylamino)-phosphonium
^{t}Bu	*tert*-butyl
° C	degrees Celsius
CD	circular dichroism
CLV3	CLAVATA3
COSY	correlation spectroscopy
*m*CPBA	*meta*-chloroperoxybenzoic acid
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DIEA	*N,N*-diisopropylethylamine
DFT	density functional theory
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DTBMP	di-*tert*-butyl methyl pyridine
E. Coli	*Escherichia coli*

EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
ESI	electrospray ionization
Fmoc	9-fluorenylmethoxycarbonyl
Gal	galactose
GlcNAc	*N*-acetyl glucosamine
GPI	glycosylphosphatidylinositol
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple bond coherence
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRGP	hydroxyproline-rich glycoproteins
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum correlation
Hyp	*trans*-4-L-hydroxyproline
Hyp-PS	hydroxyproline polysaccharide
IAD	intramolecular aglycon delivery
IgE	Immunoglobulin E
IIDQ	*N*-isobutyloxyl-carbonyl-2-isobutyloxy-1,3-dihydroquinone
KLH	keyhole limpet hemocyanin
MA	mixed anhydride
Me	methyl

MS	mass spectrometry
NaH	sodium hydride
nArt v 1	natural Art v 1
NIS	*N*-iodosuccinimide
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
NPT	nasal provocation test
PMA	phosphomolybdic acid
PMB	*para*-methoxybenzyl
PMBC	peripheral blood mononuclear cells
ppm	parts per million
PPI	polyproline 1
PPII	polyproline 2
PRP	proline-rich proteins
PTM	post translational modificiation
PyBOP	benzotriaozol-1-yloxytri(pyrrolidino)phosphonium hexafluorophosphate
PyBroP	bromotris(pyrrolidino)phosphonium hexafluorophosphate
R_f	retention factor
rArt v 1	recombinant Art v 1
RAST	radioallergosorbent test
rt	room temperature
s	singlet
SPT	skin prick test
q	quartet

Ser	serine
SPPS	solid phase peptide synthesis
TBAF	tetra-*n*-butylammonium fluoride
TBAH	tetra-*n*-butylammonium hydroxide
TBTU	*O*-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate
TCL	T-cell lines
TF	Thomsen-Friedenreich
Tf_2O	triflic anhydride
TFA	trifluoroacetic acid
TFFH	tetramethylfluoroformamidinium hexafluorophosphate
THF	tetrahydrofuran
TLC	thin layer chromatography
TOF	time of flight
UV	ultraviolet

ABSTRACT

The major allergen of mugwort pollen, Art v 1, is a significant contributor to hay fever in Europe and North America. A notable motif in Art v 1 – characterized by clusters of contiguous β-arabinosides of hydroxyproline – was found to be a key recognition element for antibodies generated in response to the natural protein. This dissertation details the synthesis of oligomers of β-arabinosides of hydroxyproline and the search to establish the minimal carbohydrate epitope of Art v 1.

The key issue pertaining to the formation of glycosidic bonds is the α/β selectivity at the anomeric carbon. To this end, using a 2,3,5-*O*-benzyl-1-thio-α-L-arabinofuranoside donor, we were able to obtain the Ara-Hyp monomer in 60% yield with 4:1 β:α selectivity using silver triflate and *N*-iodosuccinamide as activators.

A dimer of β-Ara-Hyp was prepared by deprotection of the *N*- and *C*- termini of the β-Ara-Hyp monomer respectively, after which peptide coupling of the two compounds was performed using HATU as coupling reagent to give the product, Boc-([β-L-Ara*f*]Hyp)$_2$-OAll, in 60% yield. Similar approaches were employed using a [2+1] or [1+2] fragment condensation strategy to produce the trimer, Boc-([β-L-Ara*f*]Hyp)$_3$-OAll, in 35% yield. The tetramer, Boc-([β-L-Ara*f*]Hyp)$_4$-OAll was produced using a [2+2] strategy in 49% yield.

We've installed terminal amides on the oligomers to best mimic the extended peptide found in the natural allergen. Production of oligomer-specific building blocks (Ac-([β-L-Ara*f*]Hyp)-OMe, Boc-([β-L-Ara*f*]Hyp)-NHMe) allowed a more convergent synthesis towards the end-capped oligomers. With this strategy, end-capped dimer, trimer, and tetramer were synthesized by fragment condensation in 48%, 35%, and 15% respectively. The end-capped glycopeptides could then be fully deprotected by global debenzylation to give the final products in quantitative yield.

Nuclear magnetic resonance and circular dichroism spectra were obtained for all synthetic glycopeptides. Analysis of the CD spectra showed that the glycosylated proline oligomers exhibit a polyproline type II helical conformation. While CD spectrum of the monomer showed that it was unordered, the elliptical curve of dimer, trimer, and tetramer all exhibited significant PPII characteristics. Spectra obtained from ^{1}H, ^{13}C, and various 2D NMR was used for comparison with NMR data taken from the natural allergen.

CHAPTER 1: INTRODUCTION

1.1 Carbohydrates in Biology

It has been shown over the past couple of decades that carbohydrates play a more important role in biology[1] than previously appreciated. Sugars are found in abundance in nature as biopolymers. Sugars can exist as oligosaccharides, polysaccharides, and/or glycosides in natural products, many of which display important biological activity. Many carbohydrates have specific roles in biological processes ranging from signal transduction[2] to immune response[3].

The functions of carbohydrates in living organisms are diverse. For example, heparan sulphate (Figure 1.1), a linear polysaccharide in proteoglycans, is found on the plasma membrane of all animal cells and regulates a wide variety of biological activities.[4] Gene mutation leading to the expression of modified proteoglycans has been implicated in conditions including rib malformations, craniofacial defects, and eye and lens defects.

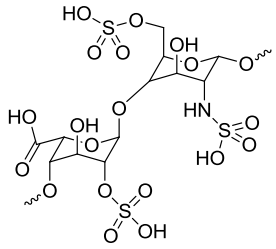


Figure 1.1 Structure of heparan sulfate subunit

Over 500 *N*-acetylglucosamine (Figure 1.2) protein conjugates are involved in almost all aspects of cellular function.[5] Both hypo-and hyper-glycosylation of these proteins have been associated with disease. For example, altered glycosylation of proteins due to nutrient excess and/or stress has been associated with glucose toxicity,[6] a.k.a. type-2 diabetes. Reduced glycosylation leading to the hyperphosphorylation of tau proteins may also affect neuronal

function. The hyperphosphorylated tau proteins aggregate into neurofibrillary tangles, one of the proposed mechanisms in the onset of Alzheimer's disease.[7]

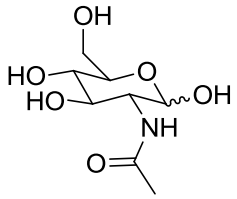


Figure 1.2 *N*-acetyl glucosamine (GlcNAc)

Advances in glycobiology and glycochemistry have enabled the development of carbohydrate-based experimental therapeutics for a variety of diseases, including HIV and cancer.[8] Carbohydrate-based tumor antigens that induce only weak immunological responses have been successfully conjugated to carrier proteins to illicit a more powerful immune response.[9] Advances in glycopeptide assembly have led to synthetic antitumor vaccines such as G_{D3} (Figure 1.3) that are conjugated to keyhole limpet hemocyanin (KLH), a well-known carrier protein.

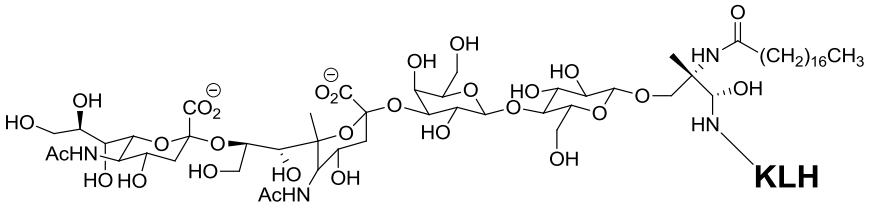


Figure 1.3 G_{D3}-Protein conjugate vaccine for melanoma

Various pathogenic bacteria are coated with polysaccharides, glycoproteins, and/or glycolipids which can be targeted by carbohydrate-rich protein-conjugated antibacterial vaccines.[10] Vaccines against *Streptococcus pneumonia*, *Neisseria meningiditis*, and *Salmonella typhi* are now commercially available.

Carbohydrate-based antiparasitic vaccines are also in development based on the unique glycoconjugates found on the surface of many parasites.[11] *Plasmodium falciparum* is the pathogenic parasite responsible for malaria, a disease which kills more than two million people per year.[12] *P. falciparum* expresses large amounts of glycosylphosphatidylinositol (GPI) on the surface of its cells. This glycolipid is responsible for the activation of the inflammatory nature of malaria. A synthetic hexasaccharide GPI (Figure 1.4) conjugated to a carrier protein was administered to malaria-infected mice. The mortality rate of the infected mice was reduced to ten percent.[13]

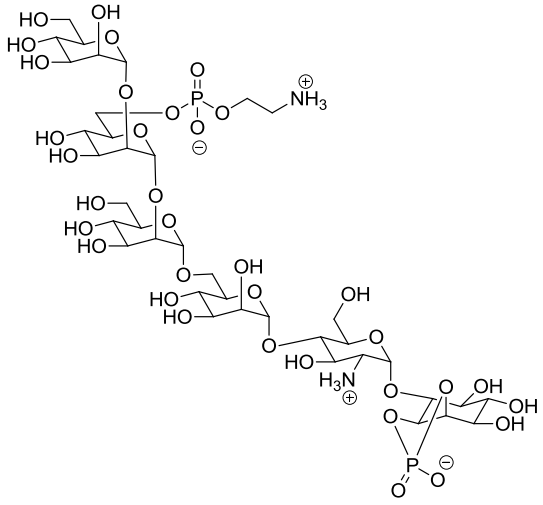


Figure 1.4 Synthetic hexasaccharide GPI

1.2. Hydroxyproline-Rich Glycoproteins

The focus of this dissertation, Art v 1, is a hydroxyproline-rich glycoprotein (HRGP). Glycosides of hydroxyproline (Hyp) in the plant cell wall matrix were originally discovered by Lamport and co-workers in the 1960s.[14] These glycoproteins and proteoglycans are widely distributed in the plant kingdom[15] with functions associated with growth, cell differentiation, and plant defense.[16] The HRGPs can be divided into four groups:

1) Extensins - Hyp-rich glycoproteins with repeating sequences such as $Ser(Hyp)_4$ and are highly glycosylated with oligosaccharides of arabinose.

2) Arabinogalactan proteins (AGPs) - typically contain arabinogalactan (Ara-Gal) chains that are attached to the protein via a Gal-Hyp linkage.

3) Solanaceous lectins - Hyp-rich lectins that consist of a carbohydrate-binding domain and an extensin-like domain.

4) proline-rich proteins (PRPs) - a broad classification of molecules that are rich in Pro/Hyp, but which cannot be classified as any of the previous three groups.

While the HRGP can be divided into groups, there are no distinct boundaries between them. In fact, Art v 1 draws characteristics of all four HRGP groups.

1.3 Art v 1: the Major Allergen of Mugwort

Artemisia vulgaris (Figure 1.5) is a widespread weed that belongs to the *Asteraceae* family. The plant is native to temperate Europe, Asia, parts of North Africa, and has been naturalized in North America. This species is known by several common names, amongst them are chrysanthemum weed, wild woodworm, felon herb, and mugwort, and typically blooms from July to September. The pollen of this plant is a major contributor to hay fever (*allergic rhinitis*) in late summer to early fall. Art v 1, the major allergen of mugwort, is recognized by up to 95% of mugwort pollen-sensitized patients.[17] In Europe, mugwort pollen affects up to 14% of all patients with pollinosis.[18] In industrialized countries of the world, Immunoglobulin E (IgE) mediated allergy affects more than 40% of the population.[19]

Figure 1.5 *Artemisia vulgaris* plant

A modular glycoprotein, Art v 1 has a tadpole-like structure that is comprised of two major domains known as the “head” and “tail” domains. The head domain is similar to protein sequences found in plant defensins. This globular domain, comprised of amino acids residues 1-55 (Table 1.1), is cysteine-rich and stabilized by disulfide bonds. The tail domain, comprised of amino acid residues 56-108 (Table 1.2), is the proline-rich domain. This section contains about 20 proline residues and is heavily hydroxylated and glycosylated. The prolyl domain facilitates protein folding[20] and influences the conformation of the globular domain.[21]

Table 1.1 Amino acid residues in the “Head” domain

1-10	11-20	21-30	31-40	41-50	51-55
AGSKL**C**EKTS	KTYSGK**C**DNK	K**C**DKK**C**IEWE	KAQHGA**C**HKR	EAGKES**C**F**C**Y	FD**C**SK

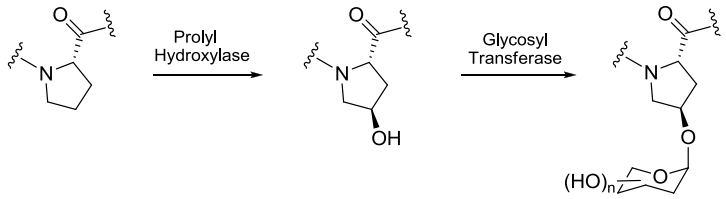
Table 1.2 Amino acid residues in the “Tail” domain

56-60	61-70	71-80	81-90	91-100	100-108
S**PP**GA	T**P**A**PP**GAA**PP**	**P**AAGGS**P**S**PP**	ADGGS**PPP**A	DGGS**PP**VDGG	S**PPPP**STH

.

1.4 Natural Versus Recombinant Art v 1

During the primary post-translational modification (PTM) of the polyproline domain of HRGPs, most of the proline residues are hydroxylated by a prolyl-4 hydroxylase (Scheme 1.1). The hydroxyprolines are further modified by subsequent *O*-glycosylation with some type of carbohydrate molecule(s) during the secondary PTM. The carbohydrates in natural Art v 1 constitute about 30-40% of the mass of the molecule.



Scheme 1.1 Two sequential post translational modifications

The cDNA sequence of the Art v 1 glycoprotein was determined.[17] Natural Art v 1 (nArt v 1) was characterized and expressed in *Escherichia coli* and produced as the recombinant allergen (rArt v 1). The major difference between the natural and recombinant allergen is that rArt v 1 lacks the post translational modifications of the proline rich domain. Any differences found in biological reactivity of the two allergens could be attributed to the carbohydrate moiety of the natural versus recombinant allergen.

1.5 Two Novel *O*-Glycans in the Polyproline Domain

The hydroxyproline-rich domain of Art v 1, also known as the tail section, contains amino acid residues 56-108. Post translational modification of the tail section gives rise to two novel *O*-glycans that have been described by Leonard *et al*.[22] Through the use of high field NMR, in conjunction with chemical and enzymatic degradation and mass spectrometry, Leonard reported the existence of a new carbohydrate determinant in Art v 1. The three major peaks of the gel filtration chromatogram of alkali-degraded nArt v 1 are shown in Figure 1.6.

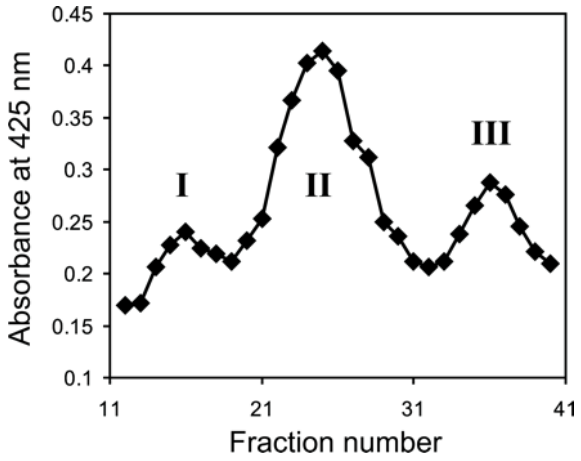


Figure 1.6 Gel filtration chromatogram of alkali-degraded nArt v 1. Reprint with permission from Journal of Biological Chemistry

The first and smallest peak (**I**) of the gel filtration chromatogram was attributed to incompletely digested material (Figure 1.6). The second peak (**II**) was most intense and attributed to hydroxyproline-linked arabinogalactan, which the authors termed Hyp-polysaccharide (Hyp-PS). Natural Art v 1 was found to contain an 11.4:1 ratio of arabinose and galactose residues. Analysis of Hyp-PS showed that it contained arabinose and galactose residues in a 5.5:1 ratio, about half of all arabinose residues in the glycoprotein. Limited acid hydrolysis of Hyp-PS saw cleavage of the arabinose units while leaving the trigalactosyl-Hyp intact with a calculated [M+Na]+ ion mass of 640 Da. When Hyp-PS was treated with α-*L*-arabinosidase, similar results were found indicating that Hyp-PS composed of a galactosyl-core with α-*L*-arabinose residues attached. Hyp PS has a β-1,3-linked galactan backbone with side chains of β-1,6-linked units similar to type II arabinogalactans (arabino-3,6-galactan),[23] but with differing patterns of substituted arabinose residues. Leonard *et al.* proposed that this be termed a type III arabinogalactan polysaccharide (AGP). Mass spectrometry indicated that Hyp-PS has isoforms that contain 5-28 α-linked arabinofuranose residues in the positions indicated in Figure 1.7. β-Glucosyl Yariv reagent (Figure 1.8), a synthetic phenyl glycoside that specifically binds to arabinogalactan polysaccharides (AGP),[24] was used to precipitate natural Art v 1. This positive test confirms that Hyp-PS is an AGP.

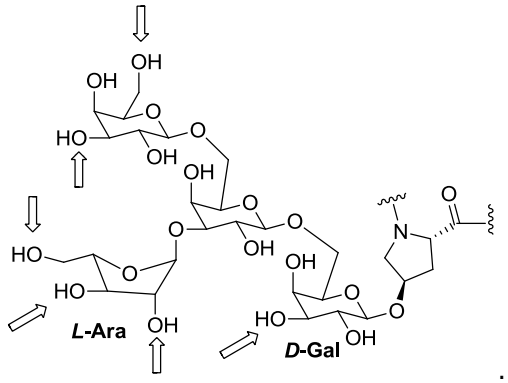


Figure 1.7 The unusual arabinogalactan (Hyp PS); arrows indicate potential sites for further arabinosylation

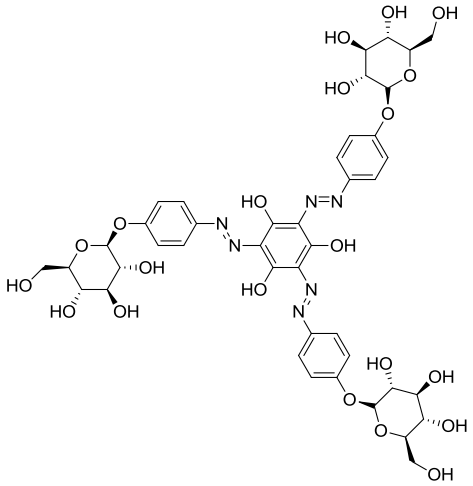


Figure 1.8 β-glucosyl Yariv reagent

Type II arabinogalactans have known to be immunogenic with monoclonal antibodies binding to arabinose-containing epitopes.[25] Surprisingly, Hyp-PS bound very weakly to antibodies from the sera of mugwort-allergic patients.[22] A similar glycoprotein from *Phleum pratense* also disappointed investigators by its insignificant binding to IgE of patients.[26]

The final peak (**III**) in the gel filtration chromatogram of alkali degraded nArt v 1 represents β-*L*-arabinoside of hydroxyproline (β-*L*-Ara*f*-Hyp) (Figure 1.6). Initially, it was thought that the carbohydrate domain of Art v 1 comprised only of α-arabinose and β-galactose residues. However, upon treatment of nArt v 1 with α-arabinofuranosidase and β-galactosidase,

the product had a calculated mass much larger than the total peptide mass of the carbohydrate region of nArt v 1 (Figure 1.9). The authors calculated that about 2.5 kDa of unaccounted mass still remained in the enzymatically treated glycoprotein. This mass was eventually attributed to 16-17 arabinose residues that were resistant to the α-arabinosidase.

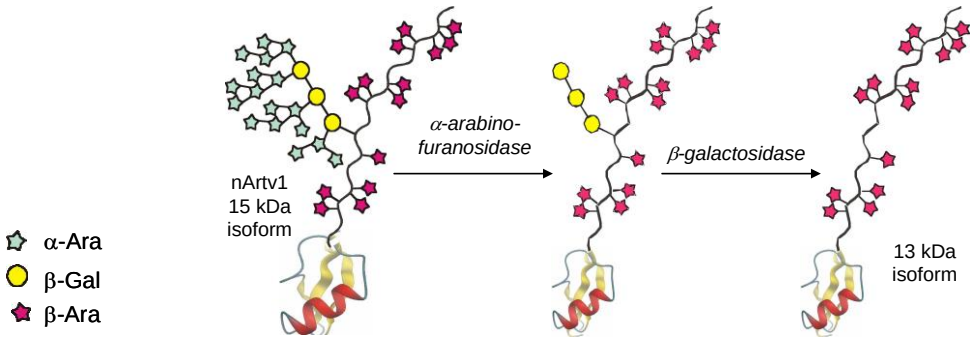


Figure 1.9 Enzymatic degradation of Art v 1.

Proton and two dimensional NMR data suggested that the 16-17 residues of "dark matter" were single β-arabinofuranosides linked to hydroxyproline (Figure 1.10a). This novel *O*-glycan does occur as a monomer, but anywhere from two to four adjacent β-arabinosylated prolines may be present (Figure 1.10b, **1**). Unlike other well-known HRGPs having the Ser-Hyp_4 motif, no oligo-arabinosides were found in Art v 1. This second new type of *O*-glycan *did* react with antibodies from the sera of mugwort-allergic patients. As reported earlier, comparison of the recombinant and natural allergen showed that a number of patients responded only to the natural Art v 1.[27] This means that the post translational modifications, namely proline hydroxylation and subsequent β-arabinosylation, are influencing the conformation of the epitopes of the mugwort allergen. While the exact mechanism of the stabilizing effects of arabinoglycosylated prolines are yet unclear, it has been implicated that β-arabinosides in the polyproline-domain does influence the allergenicity of Art v 1.[21]

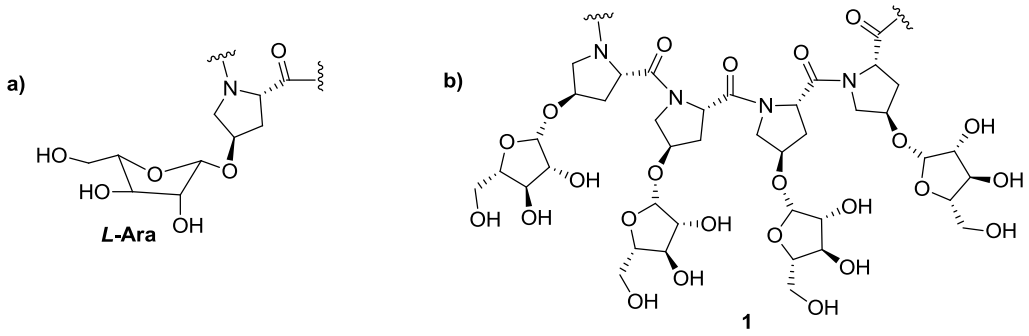


Figure 1.10 a) The single β-L-Ara-Hyp motif, b) Tetramer of Hyp-β-arabinofuranoside

1.6 Immunological Studies

1.6.1 *In vitro* and *in vivo* testing of Art v 1

T-cells, or T-lymphocytes, are a specialized type of white blood cell that play a central role in cell-mediated immunity. Initial testing showed that the post-translational modifications did not influence T-cell recognition of Art v 1.[17] In a related study by Jahn-Schmid *et al.*, peripheral blood from eighteen mugwort pollen-sensitized patients was collected based on case history and positive *in vitro* and *in vivo* tests. These patients were found to have IgE that recognized both nArt v 1 and rArt v 1. For this patient group, the nArt v 1 and its recombinant form elicited similar T-cell responses in peripheral blood mononuclear cells (PBMC) and in allergen-specific T-cell lines (TCL).[28]

T-cell epitopes were determined by TCL against both the natural and recombinant allergen. The T cell proliferation assays utilized thirty-three overlapping 12-mer peptides that spanned the entire amino acid sequence of rArt v 1. Out of seventeen patients, fourteen recognized an Art v 1 epitope at amino acids 22-36 in the cysteine rich region of Art v 1. Five patients recognized an epitope at amino acids 43-54, also in the cysteine domain. Only two of seventeen patients exhibited multiple dispersed epitopes, a rather low number in comparison to

known epitopes of other pollen allergens. A follow up study by this group reported that the single T cell epitope Art v 1_{22-36} is associated with the expression of HLA-DRB1*01.[1]*, [29]

In the first clinical study of mugwort allergens, Schmid-Grendelmeier and co-workers investigated the allergenicity of nArt v 1 and rArt v 1 by both *in vitro* and *in vivo* experiments.[30] Thirty-two patients with mugwort pollen allergy (17 female, 15 male; 16-43 years old) and 10 control subjects (7 female, 3 male; 21-41 years old) were included in this study. The thirty-two mugwort allergic patients were selected based on having a clinical history of recurrent rhinitis, a positive skin prick test (SPT) response to mugwort extracts, and increased IgE levels to mugwort pollen.

The *in vitro* results agreed with the findings of Jahn-Schmid and co-workers. It was reported that both nArt v 1 and rArt v 1 alone were able to induce T-cell proliferation in mugwort-sensitized patients. The proliferative responses of PBMCs to rArt v 1 and nArt v 1 were comparable, the only difference being that the recombinant form required longer incubation periods to induce lymphocyte proliferation.

The *in vivo* tests, however, showed that the recombinant allergen elicits a lower SPT and nasal provocation test (NPT) reactivity than the natural allergen. While rArt v 1 was still able to elicit positive SPT and NPT, the amount of rArt v 1 required was significantly higher than that for nArt v 1. The recombinant allergen showed a decrease in the size of the wheals induced in the SPT, while having a reduced response compared to that of its natural counterpart in the NPT.

[1]*Human Leukocyte Antigen (HLA) is the name of the major histocompatibility complex (MHC) in humans. MHC mediates the interaction of leukocyte (white blood cells) with other leukocytes or body cells.

1.6.2 IgE recognition of Art v 1

Immunoblots, radioallergosorbent tests (RAST), and enzyme-linked immunosorbent assays (ELISA) were used to evaluate the IgE binding properties of natural and recombinant Art v 1.[17] Immunoblots and RAST showed that two groups of patients exist: one group that exhibits similar IgE recognition of nArt v 1 and rArt v 1, and a second group that showed significantly lower or no reactivity to the recombinant allergen. ELISA experiments showed that rArt v 1 only caused a partial (30%) inhibition of IgE binding to nArt v 1.

Oberhuber and co-workers published a paper in 2008 detailing the analysis of IgE binding profiles in a group of mugwort-allergic patients.[27] Sera from 100 pediatric mugwort allergic patients (62 males, 38 females; 1-19 years old) were tested. Patients all showed hypersensitivity to mugwort based on a SPT from mugwort pollen extract and a positive RAST.

In order to evaluate IgE binding activity, ELISA experiments were performed with purified nArt v 1 and rArt v 1. The natural allergen was recognized by the serum of 79 of the 100 patients. As for the recombinant allergen, only 39 patients' sera recognized the protein, a 50% drop in allergen recognition as compared to nArt v 1.

More recent work has shown that glycosylation of the natural Art v 1 protein contributes to the thermal stability of the allergen in that it aids in the complete refolding of the glycoprotein after heat denaturization, something that the recombinant allergen could not replicate.[20] Razzera and co-workers reported, after comparing NMR chemical shifts of the recombinant and naturally glycosylated Art v 1, that the carbohydrates in the polyproline domain affect the defensin domain in the natural molecule (Figure 1.11).[21] These results, in combination with the findings of Himly and Oberhuber *et al*.,[17, 27] strongly suggest that, for a significant group of patients, the involvement of post-translational modification and the resulting carbohydrates are crucial in the formation of IgE binding epitopes of Art v 1.

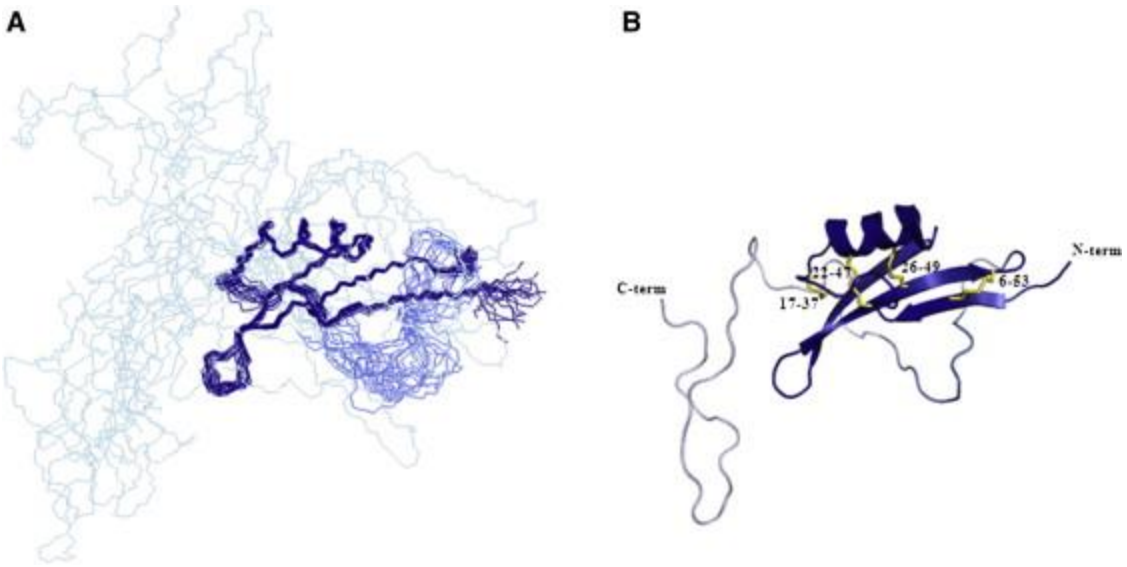


Figure 1.11 NMR solution structure of Art v 1. a) Defensin domain in dark blue, intermediate region in blue, and polyproline domain in light blue. b) Ribbon illustration of Art v 1. Reprinted with permission from Elsevier.

1.7 A Related Allergen from *Ambrosia*

Altmann and coworkers recently characterized a new allergen, Amb a 4 (Figure 1.12), from ragweed (*Ambrosia artemisiifolia*).[31] The isolated ragweed pollen protein consisted of a defensin-like domain with a 50% homology to Art v 1 (Table 1.3). The *C*-terminal hydroxyproline-rich domain contained small amounts of the single hydroxyproline-linked β-arabinoside residues also found in Art v 1. The recombinant ragweed protein reacted with the sera of more than 30% of weed pollen allergic patients.

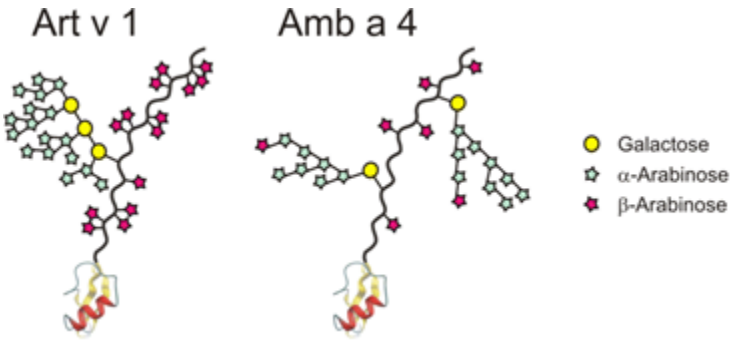


Figure 1.12 Cartoon representations of Art v 1 and Amb a 4

Table 1.3 Proline-rich domains of Art v 1 and Amb a 4.

	56	61	71	81	91	101
Art v 1	S**PP**GA	T**P**A**PP**GAA**PP**	**P**AAGGS**P**S**PP**	ADGGS**PPPP**A	DGGS**PP**VDGG	S**PPPP**STH
Amb a 4	-N**P**G**P**	**PP**GA**P**KGKA**P**	A**P**S**PP**SGGGA	**PPP**SGGEGGD	G**PPPP**EGGEGG	GGDGGGE

1.8 *Arabidopsis* CLV3 Glycopeptide: Another β-*L*-Arabinoside of Hyp

CLAVATA3 (CLV3) is a glycopeptide gene secreted from *Arabidopsis thaliana* plants.[32] The CLV3 gene is expressed in the shoot apical meristem (SAM), which continuously produces organs for the plants, and is a key component in the regulation of stem cell renewal and differentiation.[33] Overexpression in the CLV3 gene can lead to developmental retardation.[32] It has been reported that the mature CLV3 peptide found in CLV3-overexpressing *Arabidopsis* plants feature a 13 amino acid peptide.[34] This peptide features a *trans*-4-hydroxyproline at the seventh residue (Hyp7) that is attached to three L-arabinose residues via β-1,2-linkages (Figure 1.13). In testing the restrictions of stem cell activity by synthetic peptides varying in the number of carbohydrates attached to Hyp7, it was found that the biological activity increased with the length of the arabinose chain.

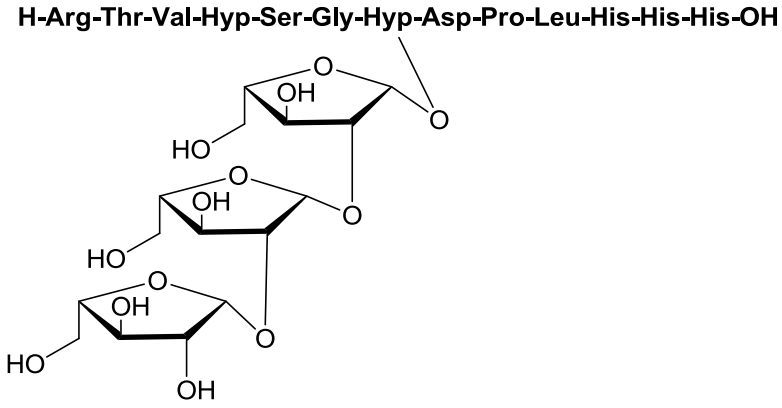


Figure 1.13 CLV3

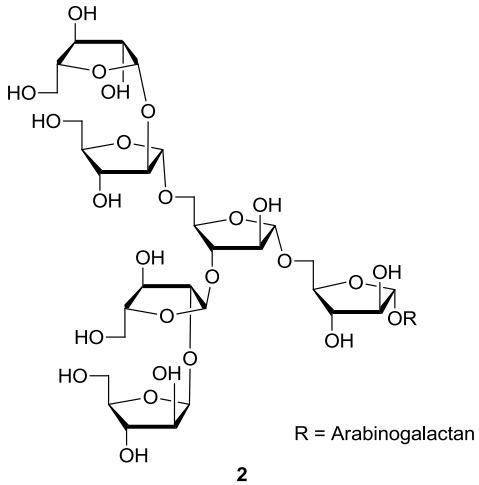
1.9 Closing Comments

Small amounts of Art v 1 have been isolated from the pollen of *Artemisia vulgaris*. The isolation and chemical characterization of the allergenic epitope of Art v 1, however, is not practical through extraction and partial degradation of the natural protein. There is much difficulty in obtaining pure compounds due to the heterogeneous nature of the glycoprotein. Chemical degradation techniques typically lead to a complex mixture of amino acids. In order to clearly identify the carbohydrate motif that contributes to the epitopes of Art v 1, homogenous compounds of monomer, dimer, trimer, and tetramer must be obtained by way of chemical synthesis. This dissertation describes our efforts to synthesize homogenous oligomers of hydroxyproline β-arabinosides. The following chapters will present the synthetic methods, challenges, and triumphs we've endured upon our path to this goal.

CHAPTER 2: SYNTHESIS OF A MONOMER OF 4-*O*-[β-*L*-ARABINOFURANOSYL]-(2*S*,4*R*)-4-HYDROXYPROLINE

2.1 Importance of Arabinosides

Both enantiomers of arabinose exist in nature. β-Linked homopolymers of *D*-arabinose can be found in the cell wall of *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Figure 2.1),[35] while the *L*-form is an important component of plant cell walls.[36] As described in Chapter 1, we are specifically interested in contiguous β-*L*-arabinofuranosides of hydroxyproline, a motif that is found in mugwort pollen. We believe this constitutes a significant allergenic epitope of the Art v 1 glycoprotein. The chemical synthesis of arabinosides, to further investigate their biological significance, has therefore become an important endeavor.



2

Figure 2.1 Arabinofuranosyl residues found in the cell wall of mycobacterium

2.2 Challenge of β-Arabinoside Synthesis

Methods for the stereoselective introduction of furanosides are not as well developed as for their pyranoside counterparts.[37] The key issue pertaining to the formation of glycosidic bonds is the α/β selectivity at the anomeric carbon. Two major factors that influence this

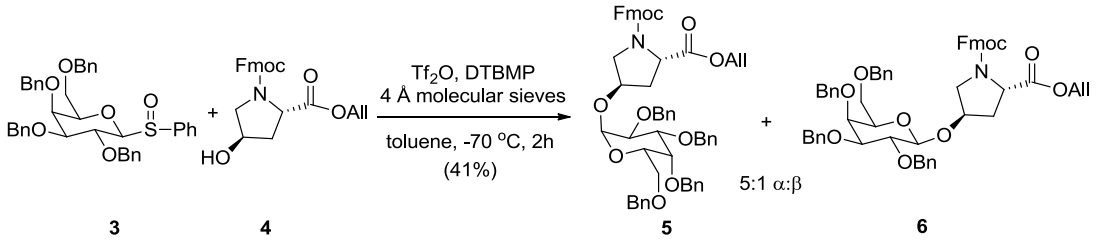
stereochemistry are highlighted in Figure 2.2 and will be discussed in more detail in the following sections.

Figure 2.2 Factors that influence stereochemistry in glycosylation. a) The anomeric effect, although strong for pyranoses, offers little selectivity for its furanose counterpart. b) Neighboring group participation favors the 1,2-*trans*-glycoside.

2.2.1 The anomeric effect

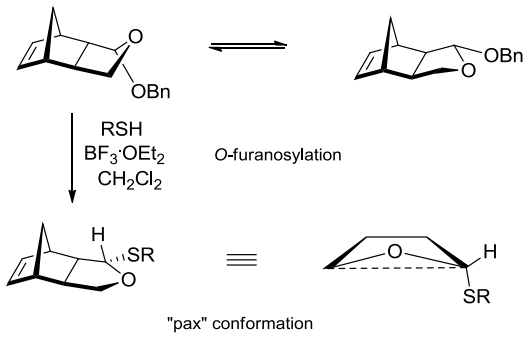
The anomeric effect is a stereoelectronic effect that describes the tendency of incoming substitutents on the anomeric carbon of a pyranose ring to prefer the axial orientation. This effect is typically used by carbohydrate chemists to control the stereoselectivity of a glycosylation reaction.

The anomeric effect is well understood and controlled for pyranoses that have a strong conformational preference for a chair (Scheme 2.1),[38] but the effect is weak for furanoses and by itself is not sufficient to influence α/β selectivity.[39] Furanoses may have up to 20 low energy twist and envelope conformations, while able to adopt an infinite number of conformations differing slightly from those ideal conformations.[40] These numerous low energy conformations can lead to many different transition states during glycosylation that does not bias the anomeric selectivity. If the conformations were more rigid, it might be possible to have more stereocontrol.



Scheme 2.1 Stereoselectivity utilizing the anomeric effect

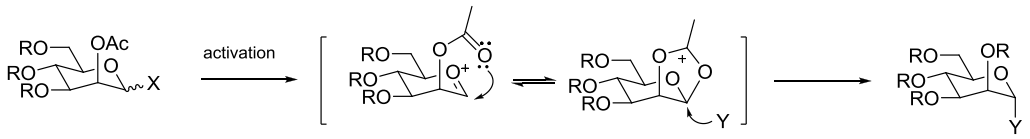
The Magnusson group published a paper in 1994 detailing the anomeric effect in conformationally restricted furanosides.[39] They chose a furanose that was fused, at C-3 and C-4, with a norbornane ring system (Scheme 2.2). The rigidity of the norbornane ring restricts the conformational flexibility of the furanose ring. With all the carbons in one plane, the only two conformations allowed would be those with the oxygen either above (^oE) or below (E_o) the plane of the ring. Upon *O*- and *S*-furanosylation of this fused ring system, they found that the anomeric substituent did indeed favor what they called the "pax" conformation, which is the pseudo-axial conformation. This work showed that with strict conformational control of the furanose ring, one can control the stereoselectivity of the furanosylation by invoking the anomeric effect.



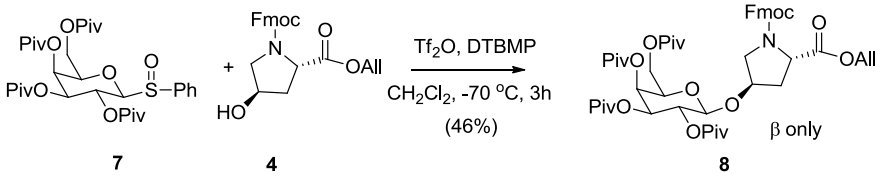
Scheme 2.2 Conformational restriction leading to increased anomeric effect in furanoside

2.2.2 Neighboring group participation

Neighboring group participation (Scheme 2.3), a phenomenon widely used to good advantage by carbohydrate chemists to control stereoselectivity at the anomeric carbon, heavily favors the formation of the 1,2-*trans* product. Scheme 2.4 shows the glycosylation of Fmoc-protected hydroxyproline allyl ester (**4**) with a sulfoxide donor **7** having a participating pivaloyl group at C-2 giving the only the *trans* product **8**.[38] Unfortunately for us, this would lead to the formation of the α-arabinoside.



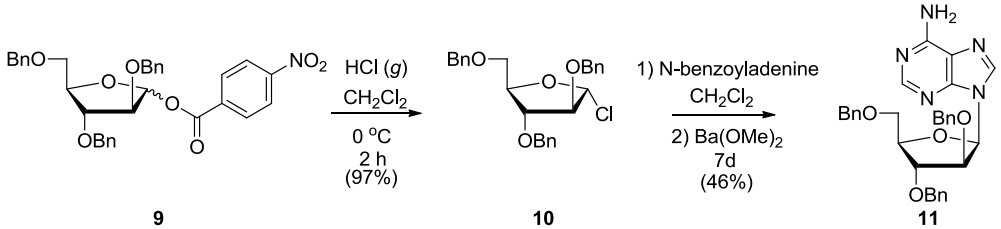
Scheme 2.3 Neighboring group participation



Scheme 2.4 Stereocontrol via neighboring group participation

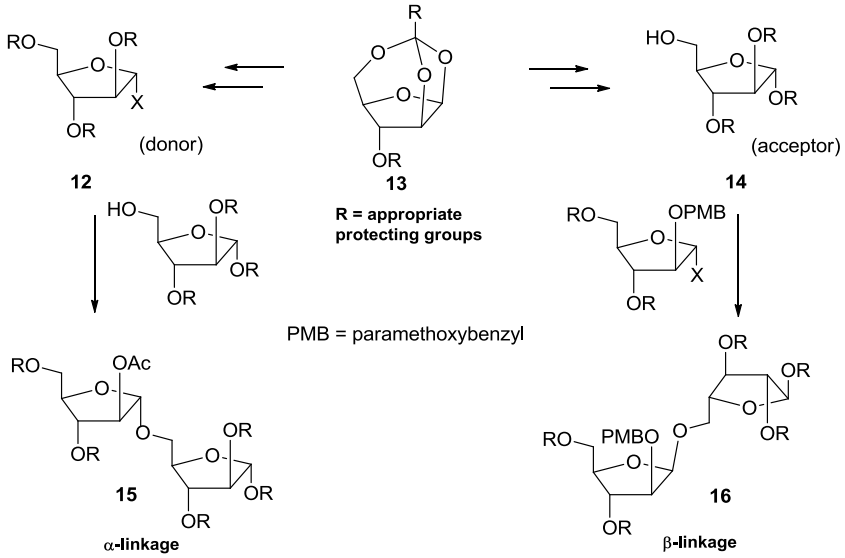
2.3 Previous Efforts to Produce β-Arabinosides

Amongst some of the earliest work in the effort to obtain 1,2-*cis*-arabinosides, Claudemans and coworkers employed glycosyl halides (e.g., **10**) which were masked at C-2 by a nonparticipating group, *viz* a benzyl ether, for the synthesis of 9-β-D-arabinofuranosyladenine (**11**) (Scheme 2.5).[41] They later reported that, mechanistically, the glycosylation exhibited both S_N1- and S_N2-type properties and, regardless of the configuration of the C-1 halide, having a nonparticipating group at C-2 gave the more stable *trans* ion pair which would yield the *cis*-product.[42] Furthermore, the rate of glycosylation was increased with each hydroxyl group having been protected as an "armed"[43] benzyl ether as opposed to a more electron withdrawing species.[44]



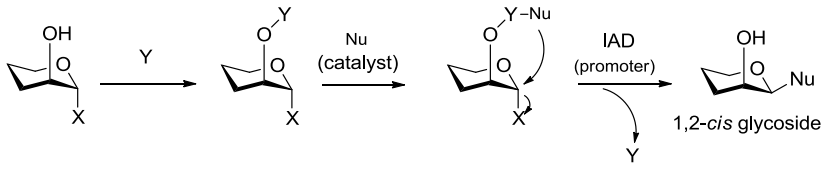
Scheme 2.5 Early efforts to obtain 1,2-*cis*-arabinosides

Prandi and coworkers published papers in 2000 showcasing 1,2,5-orthoester **13** in the synthesis of a penta-arabinofuranoside of the mycobacterial cell wall (Scheme 2.6).[45] The orthoester intermediate **13** can be converted to either a glycosidic donor **12** or acceptor **14**. The stereoselectivity of the subsequent glycosylation reaction is controlled by the protecting group at C-2. α-Arabinosidic linkages (*viz*, **15**) could be made with a participating C-2 acetate, while β-linkages (*viz*, **16**) are accessed through a *para*-methoxybenzyl (PMB) ether at C-2.



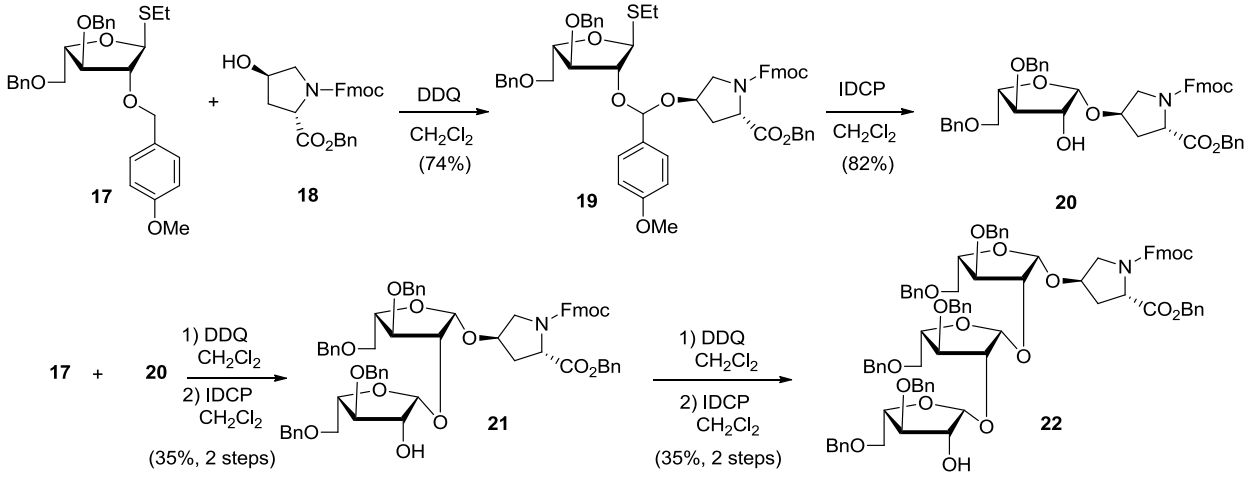
Scheme 2.6 Use of 1,2,5-orthoester as donor and acceptor

Intramolecular aglycon delivery (IAD) is a more recent approach to synthesize 1,2-*cis*-glycosides (Scheme 2.7). In this method, the glycosyl acceptor is first tethered to the protecting group at C-2. Activation of the anomeric leaving group, along with subsequent formation of the oxacarbenium ion, allows for the delivery of the tethered nucleophile from the same face to form the new glycosidic bond. Prandi's use of a PMB ether (Scheme 2.6) at C-2 to facilitate an intramolecular aglycon delivery to give only the β-linked polysaccharides is an example of this strategy.



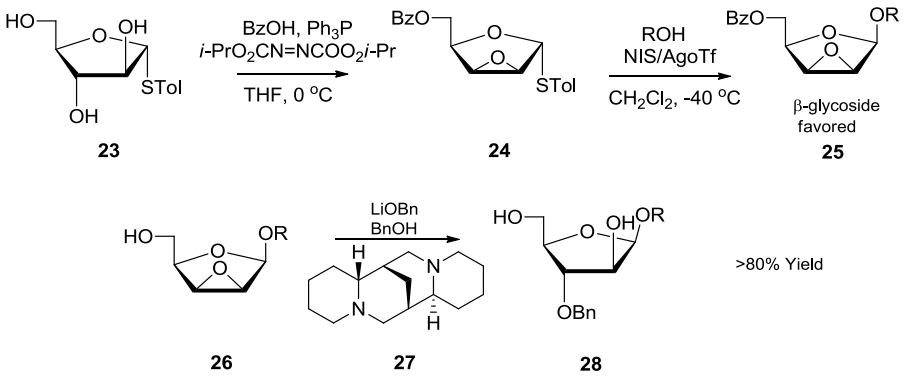
Scheme 2.7 Intramolecular aglycon delivery

More recently, Shinohara *et al.* reported on the synthesis of *Arabidopsis* CLV3 glycopeptide utilizing IAD for the installation of its β-arabinofuranosidic linkages.[34] The CLV3 peptide is a 13 amino acid glycopeptide containing a β-*L*-triarabinosylated hydroxyproline (Scheme 2.8, **22**). The three arabinose residues are linked to one another via β-1,2-linkages. *N*α-Fmoc hydroxyproline benzyl ester (**18**) and thioglycoside donor **17** were treated with DDQ to give the mixed acetal **19** in 74% yield. Activation of the anomeric thio leaving group gave the β-glycoside **20** in 82% yield. This process is repeated twice more to give the protected Hyp triarabinofuranoside **22**. Incorporation of the synthetic glycopeptide into the 13 amino acid chain was done by solid phase peptide synthesis. Comparison of the mono-, di-, and triarabinosylated CLV3 peptides showed that increased arabinosylation corresponded to increased biological activity.



Scheme 2.8 Synthesis of CLV3 glycopeptide utilizing IAD

Lowary's group showed that β-arabinofuranosides could be made with high stereoselectivity through a 2,3-anhydro sugar intermediate **24** (Scheme 2.9).[46] Transformation of a thioglycoside triol **23** into a 5-benzoyl-2,3-epoxide donor **24** was achieved in one step. Subsequent activation of the leaving group and addition of an alcohol nucleophile gave glycosides **25** with good β-selectivity. However, increasing the steric bulk of the alcohol nucleophile led to lower β-selectivity. Acceptors bearing electron withdrawing protecting groups also suffered a slight loss in selectivity. Nonetheless, upon treatment of the epoxide **26** with LiOBn in benzyl alcohol and sparteine (**27**), the β-arabinoside **28** could be obtained in desired regioselectivity and great yield.



Scheme 2.9 stereoselective β-arabinosylation through a 2,3-anhydro sugar intermediate

Boons and co-workers devised a practical approach to the stereoselective synthesis of β-*L*-arabinofuranosides.[47] Bearing in mind that furanosides have many low energy conformations, the Boons group sought to lock the thioglycoside donor into a conformation that would favor nucleophilic attack from the β-face. Using density functional theory (DFT) quantum mechanical calculations, the optimized geometries of the arabinofuranosyl oxacarbenium ion were found to be the ^3E and E_3 conformations (Figure 2.3a). The E_3 conformation permits a staggered relative orientation of the substituents for the 1,2-*cis* attack and thus would favor the formation of a β-glycoside. In order to apply the principle of conformational restraint, they employed a 3,5-*O*-di-*tert*-butylsilane protecting group that gave a [6,5] bicyclic molecule (Figure

2.3b), in which the 6-membered ring would be a chair, locking the furanose ring in the desired E_3 conformation.

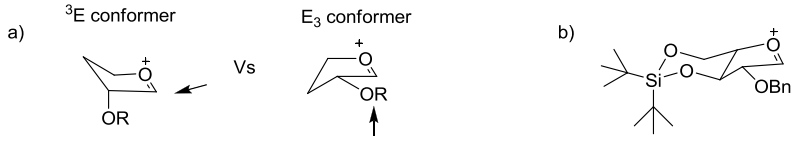
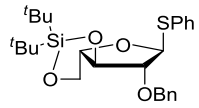
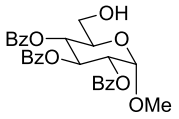
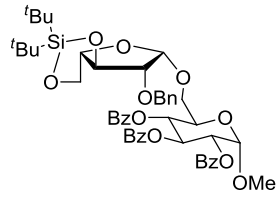
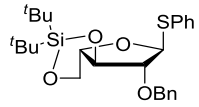
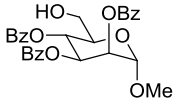
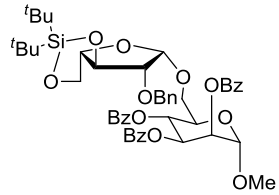
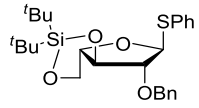
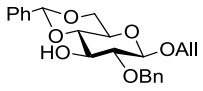
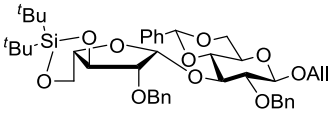
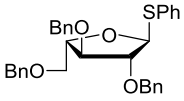
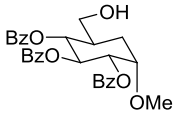
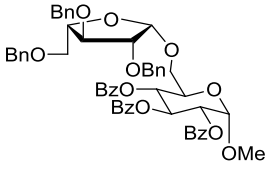
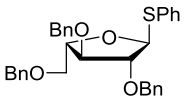
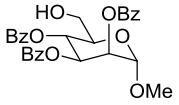
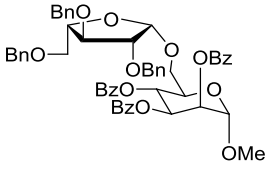


Figure 2.3 a) In the ^{3}E conformation, the C-2 substituent is axial, resulting in steric interactions that favor alpha attack. The E_3 conformation allows the C-2 substitutent to be in a pseudo-equatorial orientation, favoring a beta attack at the anomeric carbon. b) C-3 and C-5 alcohols protected as a silyl acetal.

With the thioglycoside donor **29** in-hand, Boons' group investigated several different glycosyl acceptors (Table 2.1, Entries 1-3). For most pyranose derivatives with a free primary alcohol at C-6 (Entries 1 and 2), activation of the donor with NIS/AgOTf provided excellent selectivity ranging from 15:1 β/α to 100% β, along with good chemical yields, ranging from 82% to 95%. In the one case where the overall yield was only 69%, β-selectivity was 100% (not shown). Pyranoses with a secondary C-3-OH acceptor (*e.g.*, Entry 3) were also studied under the same activation conditions. Stereoselectivity in these reactions slipped to 8:1 β/α, while the yields were in the mid-80's.

Table 2.1 Selected examples of glycosylation with *L*-arabinosyl donors. Reagents and conditions: NIS/AgOTf, DCM, -30 °C

	DONOR	ACCEPTOR	β/α ratio / YIELD	GLYCOSIDE
1	 **29**	 **30**	β/α = 15/1 91%	 **31**
2	 **29**	 **32**	β only 94%	 **33**
3	 **29**	 **34**	β/α = 8/1 82%	 **35**
4	 **36**	 **30**	β/α = 3/1 88%	 **37**
5	 **36**	 **32**	β/α = 2/1 85%	 **38**

In order to prove that the conformationally restricted donor **29** was responsible for the excellent β-selectivity, the same primary alcohols mentioned above were reacted with a 2,3,5-tri-*O*-benzylated thioglycoside donor **36** (Entries 4 & 5). Although the chemical yield remained good, as expected, the β/α ratio dropped significantly (Entries 1 vs 4, 2 vs 5). This provides empirical proof of the effectiveness of conformational control in the synthesis of *β*-arabinofuranosides.

Crich and co-workers simultaneously developed a similar approach to assemble the *β*-D-arabinofuranothioglycosyl donor, but first investigated a 3,5-*O*-benzylidene protected donor (**39**, Figure 2.4) since the six membered ring had provided good conformational control in the case of pyranoses (e.g., glycosylations with **40**).[48] Unfortunately, in the case of furanosides, donor **39** was difficult to synthesize and found to not be as stable as the silylene protected donor. In addition to being less stable, the benzylidene-protected thioglycoside **39** gave rise to lower β:α selectivity and typically poor yields.[49]

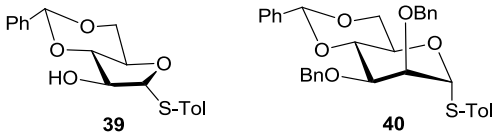
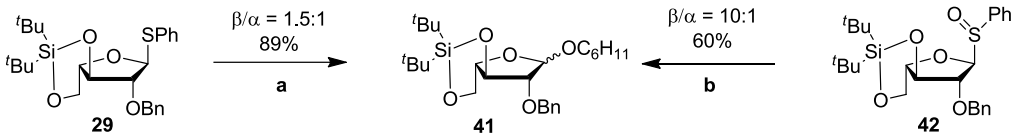


Figure 2.4 Benzylidene method adapted for β-D-arabinofuranoside formation

Since a more robust protecting group was needed, Crich and coworkers chose to move ahead with the 3,5-*O*-(di-*tert*-butyl-silane) arabinofuranoside donor **29** (Scheme 2.10). The list of acceptors they employed included methanol, cyclohexanol, and pyranoses with free primary or secondary alcohols. Based on their success with the synthesis of β-thiomannopyranosides,[50] they also decided to investigate the sulfoxide method[51] for the formation of β-arabinofuranosides. It was interesting that activation of the thioglycoside donor **29** (Scheme 2.10) with NIS/AgOTf and reaction with cyclohexanol provided a β/α ratio of only 1.5:1 (**41**),

while activation of the corresponding sulfoxide donor **42** with Tf_2O and reaction with cyclohexanol provided a β/α ratio of 10:1.



Scheme 2.10 Sulfide and sulfoxide donors. a) cyclohexanol, NIS/AgOTf, CH_2Cl_2 -30 °C → RT, b) Tf_2O -78°C, 30 min, then cyclohexanol

Other activators were also employed, but in the end the NIS/AgOTf combination employed by Boons and co-workers still gave the best β/α ratio when it came to using the *L*-arabinofuranothioglycoside as donor. Other methods of activation that did not prove useful were believed to be less successful because the activators did not completely convert the donors into glycosyl triflates, a hypothesis that was supported by low-temperature NMR experiments.[49] A point to bear in mind is that only *L*-arabinosides were activated with NIS/AgOTf. The *D*-thioarabinosides in this study were activated in other ways.

Crich concurred with Boons' hypothesis that the intermediate in the glycosylation is most likely the oxacarbenium ion in the E_3 conformation. Since the publication of these two key papers, Lowary and co-workers have reported a crystal structure of a 3,5-*O*-(di-*tert*-butyl-silane) arabinofuranoside derivative in which the furanose ring is in an E_4 conformation (Figure 2.5).[52]

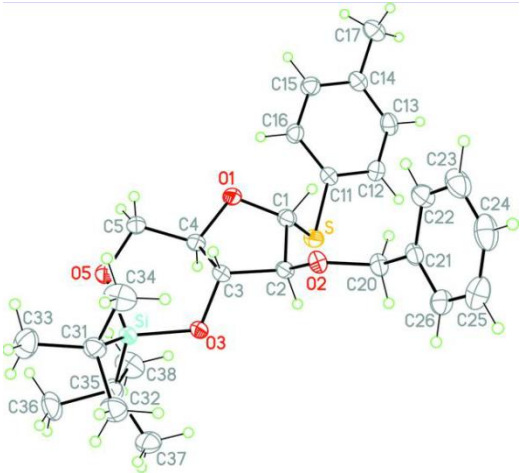
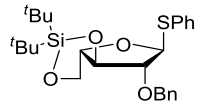
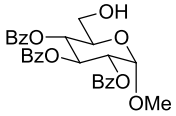
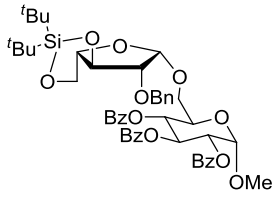
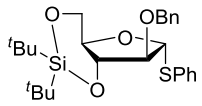
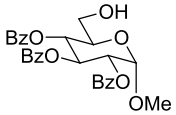
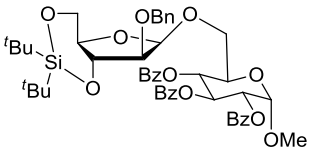


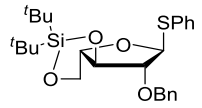
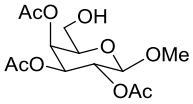
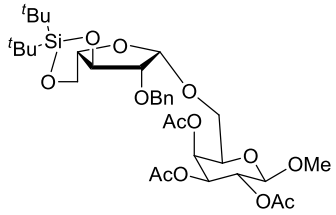
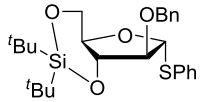
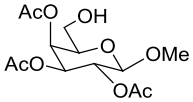
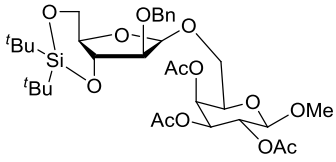
Figure 2.5 Crystal structure of 3,5-*O*-(di-*tert*-butyl-silane) arabinofuranoside derivative. Reprint with permission from IUCr.

Zhu, the first author on the 2007 paper from the Boons group, is now at University College, Dublin. The Zhu Group's goal, in a more recent paper, was to directly compare the NIS/AgOTf activation for glycosylation with *L*- and *D*-arabinofuranosyl donors (Table 2.2), something that neither the Boons nor Crich groups had done.[53] 3,5-*O*-(Di-*tert*-butyl-silane)-*D*-arabinothiofuranoside (**ent-29**) was used as the donor while several sugars with free primary and secondary alcohols were used as acceptors. Some of these acceptors were a lot like the ones that were used by Crich. Glycosylation of the primary alcohols proceeded in good yields (78% to 90%), with β/α ratios in the range of 2:1 to as high as 6:1. Glycosylation of secondary alcohols gave comparable β/α ratios, but the yield dropped slightly (not shown). Zhu concluded that, given the state-of-the-art for the synthesis of β-D-arabinosides, this can still be regarded as an efficient glycosylating agent.

Table 2.2 Selected comparisons of *L*- vs *D*-arabinofuranosyl donors.[17]

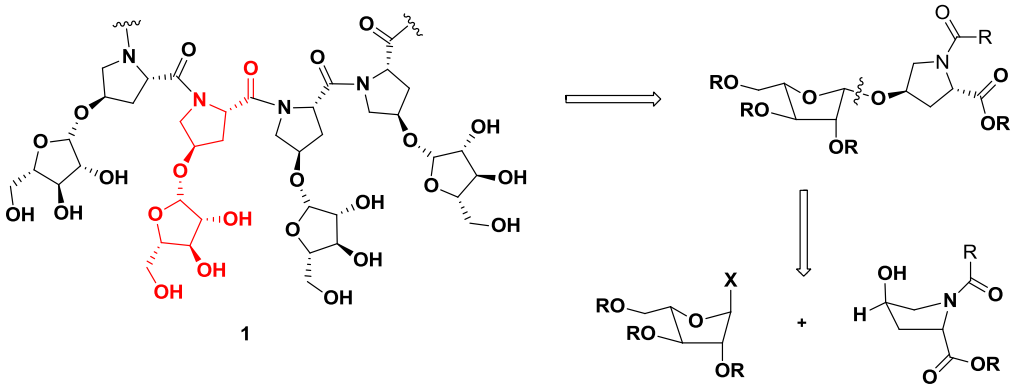
DONOR	ACCEPTOR	β:α ratio / YIELD	GLYCOSIDE
 **29**	 **30**	β:α = 15:1 91%	 **31**
 ***ent*-29**	 **30**	β:α = 8:1 83%	 **43**

(Table 2.2 continued)

DONOR	ACCEPTOR	β:α ratio / YIELD	GLYCOSIDE
 **29**	 **44**	β only 95%	 **45**
 ***ent*-29**	 **44**	β:α = 5:1 90%	 **45**

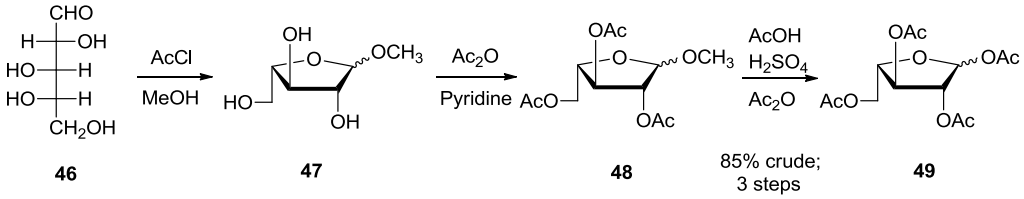
2.4 Current Investigation

Scheme 2.11 shows a tetramer of hydroxyproline-β-arabinoside (**1**). Retrosynthetic analysis shows that we must first prepare the monomer, which in turn requires us to prepare a donor and acceptor for glycosylation. In light of published results described above, we chose to adopt the Crich/Boons approach of using a conformationally restricted donor for the synthesis of our β-glycosides.



Scheme 2.11 Retrosynthetic analysis of tetramer

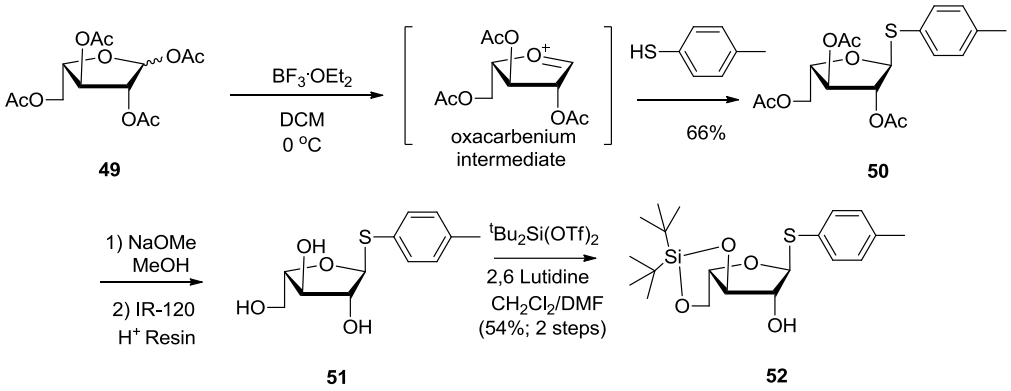
The synthesis of the donor begins with a variation on the Guthrie-Smith method[54] for the synthesis of peracylated arabinofuranose (Scheme 2.12).[55] This "general" method was also adopted by Crich. Commercially available *L*-arabinose (**46**) is present in solution as an equilibrium mixture of the linear and cyclic (both furanose and pyranose) forms. Low concentrations of HCl are generated *in situ* with acetyl chloride and methanol; these reaction conditions lock the arabinose into the methyl furanoside form. In order to obtain this kinetic product, the reaction must be stopped at three hours to prevent equilibration to the unwanted methyl pyranosides. The triol **47** was esterified with acetic anhydride in pyridine to give the 2,3,5-*O*-acylated methyl furanoside **48**. Treatment of the methyl glycoside in acetic anhydride with acetic acid, followed by sulfuric acid, gave the anomeric acetate **49**. Work up with several washes of $NaHCO_3$ is crucial as the excess acetic acid side product is extremely hard to remove. We believe compound **49** to be mostly α-furanoside due to neighboring group participation of the C-2 acetate. Unfortunately, due to the many low energy conformations of this compound,[37] the NMR spectra were complex and thus an α:β ratio was not determined.



Scheme 2.12 Peracylated arabinofuranoside synthesis

With compound **49** in hand, the next step was to form the thioglycoside donor (Scheme 2.13). Upon coordination of boron trifluoride to the oxygen of the anomeric acetate, an oxacarbenium ion is formed that undergoes nucleophilic attack by thiocresol to give thioglycoside **50**. The apparent singlet assigned to the anomeric proton confirms the formation of the α-anomer, the major product. The β-anomer (not shown in scheme) is hardly visible in NMR spectra, suggesting <10%. Temperature control during this glycosylation is crucial to limit the formation of unwanted side products. It is also important that $BF_3.OEt_2$ is added slowly to

the reaction mixture over a span of 10 to 15 minutes. The median yield for this reaction, under these optimal conditions, is 66% and reproducible on multigram scale.

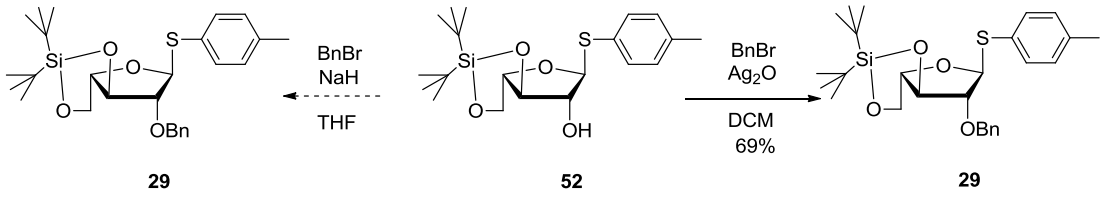


Scheme 2.13 Preparation of bicyclic silyl acetal donor

Methanolysis of the acetate esters, followed by work-up with an acidic resin, afforded compound **51**. The triol was dissolved in a 5:1 ratio of CH_2Cl_2/DMF with 2,6-lutidine as base/buffer. The dimethylformamide is essential to fully dissolve the very polar triol. After cooling to 0 °C, di-*tert*-butylsilyl-*bis*-triflate was added slowly and the reaction was allowed to warm up to room temperature overnight. While the 54% yield of silyl acetal **52** is modest, the purity of the compound obtained is evidenced by the NMR spectra.

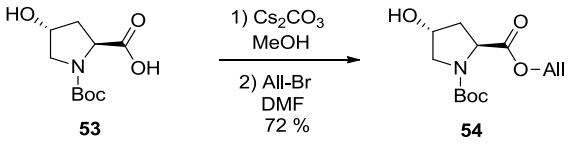
We originally attempted benzylation of the C-2 alcohol in compound **52** with sodium hydride and benzyl bromide according to Crich/Boons (Scheme 2.14),[47, 49] but this combination of reagents gave us product mixtures in which most components were more polar than the starting material. Upon isolation of these side products, it was apparent that the *tert*-butyl silyl group was being cleaved from one of the oxygens, presumably due to adventitious sodium hydroxide. Around this time, a new paper was published by the Zhu group.[53] Interestingly, a change was noted in the procedure for the benzylation reaction. Silver(I) oxide in dichloromethane is now used as the reagent/solvent in place of NaH in THF. When we contacted Zhu about the new procedure, he also cited problems with the cleavage of the silyl

acetal as the reason for the change. In this improved strategy, the silver polarizes the C-Br bond of the benzyl bromide, facilitating nucleophilic attack by the secondary alcohol of the arabinoside without subjecting the silyl protecting group to harsh, basic conditions. Compound **29** was the first glycosyl donor prepared in our lab for hydroxyproline arabinosylation.



Scheme 2.14 Benzylation of C-2 alcohol

Synthesis of the glycosyl acceptor was accomplished in one step from commercially available Boc-*L*-*trans*-4-hydroxyproline (**53**). The carboxylic acid functionality was converted to the corresponding cesium salt, which reacts with allyl bromide to give the allyl ester **54** (Scheme 2.15). We chose these protecting groups based on how we would like to deprotect them downstream. The Boc carbamate would be easily removed by trifluoroacetic acid and is orthogonal to the allyl ester, which could be removed in a mild manner by palladium (0).[56]



Scheme 2.15 Preparation of hydroxyproline acceptor

While synthesis of the hydroxyproline acceptor is straightforward, we anticipated that it would likely give us problems in glycosylation due to its poor nucleophilicity. Aside from the secondary alcohol being hindered, the pyrrolidine ring is in a Cγ-exo conformation due to gauche interactions between the substituents (Figure 2.6).[57] This places the hydroxyl functionality in a pseudo-axial orientation, favored by a hyperconjugative interaction between the axial hydrogens at Cβ and Cδ and the alcohol at Cγ.[58] These effects contribute to the overall poor nucleophilicity of the alcohol.

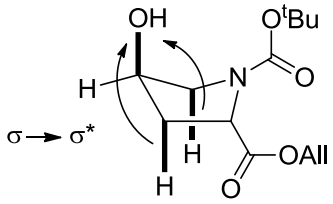
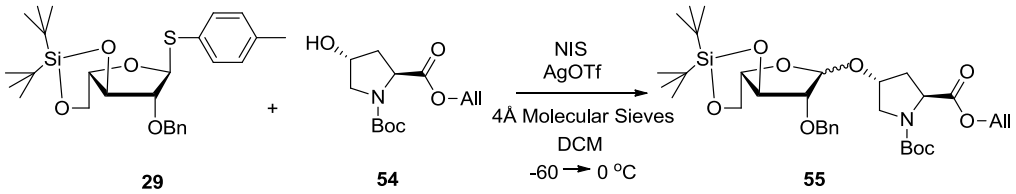


Figure 2.6 Hyperconjugation via σ→σ* interaction

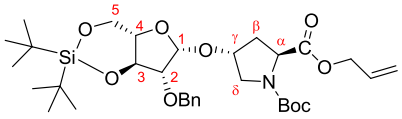
2.4.1 Glycosylation with the conformationally-restricted glycosyl donor

In our first attempt at the glycosylation of hydroxyproline we followed the procedure published by Crich, wherein the glycosyl donor was *ent*-**29** (derived from *D*-Ara) and the glycosyl acceptor was cyclohexanol (Scheme 2.16). *N*-Iodosuccinimide was used as the activator to form the oxacarbenium ion, followed by the addition of silver triflate to form the anomeric triflate. The six-membered silyl acetal ring should serve to lock the furanose into the conformation that favors β-attack. The reaction is messy, however, with a plethora of side products. Monitoring of the reaction and separation of compounds is also hampered by the fact that product **55** is not very UV active. Due to low concentrations of analyte in the test tubes after flash chromatography, anisaldehyde staining, which is generally used to detect sugar molecules, is not sufficiently sensitive. Portions of α- and β-anomers collected, even after flash chromatography, are still rather crude. We have isolated pure β-glycoside in milligram quantities by normal phase HPLC that has enabled ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR spectra to be acquired. While we do believe that there is a slight preference for the formation of the β-glycoside, the poor overall yield (~12% after HPLC) and stereoselectivity ultimately led us to investigate other glycosidic donors. We were, however, able to fully assign all protons and carbons in the NMR spectra (Table 2.3).



Scheme 2.16 Glycosylation of hydroxyproline with silyl acetal donor

NMR resonance assignments are based on the information obtained from one-pulse ^{13}C and ^{1}H experiments as well as COSY, double-quantum filtered COSY, 135° DEPT, TOCSY, and HSQC. These assignments are summarized in Table 2.3.



(a) The Proline Domain

Due to the rotational isomerization of the molecule, a 2:1 ratio of rotamers can be seen in the ^{13}C and ^{1}H NMR spectra. The ^{13}C resonances at 118.7 (118.3) ppm correlate to a single carbon, which is secondary according to the 135 ° DEPT spectrum. Correlation between this carbon and resonance 5.21-5.35 ppm leads to assignment of these signals to the terminal $=CH_2$ of the allyl ester. The COSY spectrum shows correlation of the signals arising from these olefinic CH_2 to a multiplet at 5.85-5.96 ppm, which was therefore assigned as the C-H of the monosubstituted alkene. This proton is further coupled to two protons of signals 4.55-4.69 ppm in the COSY ^{1}H spectrum, which is the allylic CH_2, as supported also by the ^{13}C 135 ° DEPT spectrum. The TOCSY spectrum shows correlation between all five protons.

Multiplets at 2.08-2.15 ppm and 2.35-2.49 ppm can be attributed to a pair of diastereotopic protons with rotational isomerization peaks identifiable by the HSQC spectrum. The chemical shifts would suggest that they are the beta protons of the proline. As expected,

the beta proton signals showed correlation with two other proton signals at 4.40 ppm and 4.29 ppm in the ^{1}H-^{1}H COSY spectrum, that were assigned to Hα (showed no other cross peak) and Hγ respectively. The Hγ showed further correlation with a pair of diastereotopic protons (3.58-3.69 ppm) (who are also correlated with each other) assigned to Hδ. The HSQC spectrum confirms that they are connected to a single carbon. TOCSY spectrum shows correlation between all six protons of the pyrolidine ring spin system.

The ^{13}C resonances at 172.7 (172.4) ppm can be assigned to the ester carbonyl functionality of the Boc group. The quaternary carbon of the *tert*-butyl group can be assigned to the ^{13}C resonances at 80.2 (80.1) ppm, and 135° DEPT spectra shows those resonances to be missing. The primary carbons of the *tert*-butyl group can be assigned to the ^{13}C resonances at 28.2 (28.4) ppm, and the HSQC spectra confirms that there are 3 protons connected to each at 1.38 (1.45) ppm on the ^{1}H spectrum.

(b) The Arabinose Domain

The anomeric proton signal can be confidently assigned to the resonance at 4.97 (4.93) ppm. The coupling constant here is 5.3 Hz, and is in the expected range for β-anomers of arabinosides (c.f., $^{3}J_{12}$= 1-3 Hz for α-arabinosides).[10] There are two doublets for H1, as a consequence of the rotational isomerization of the molecule. The anomeric signal is in correlation with one other proton at 3.90 ppm (COSY), which we assign as H2. The TOCSY spectrum shows correlation between the anomeric proton, H2, and four other signals. Two of these signals, 4.30 ppm and 3.88 ppm, show correlations to the same carbon in the ^{13}C-^{1}H HSQC spectrum; they are diastereotopic H5 protons of the arabinose ring. The remaining two signals, 3.59 ppm and 4.28 ppm, are assigned to H4 and H3 respectively.

The aromatic protons can easily be assigned to the multiplet at 7.29-7.40 ppm. HSQC data shows five distinct carbon peaks ranging from 127.7-128.3 ppm, along with a quaternary

carbon at 137.7 ppm, that directly correlate to the aromatic carbons of the benzyl group. One of the two benzyl CH_2 signals can be seen clearly in the ^1H NMR spectra with a coupling constant of 12.2 Hz. TOCSY shows correlation between all aromatic protons and even long range cross peaks with the benzyl CH_2.

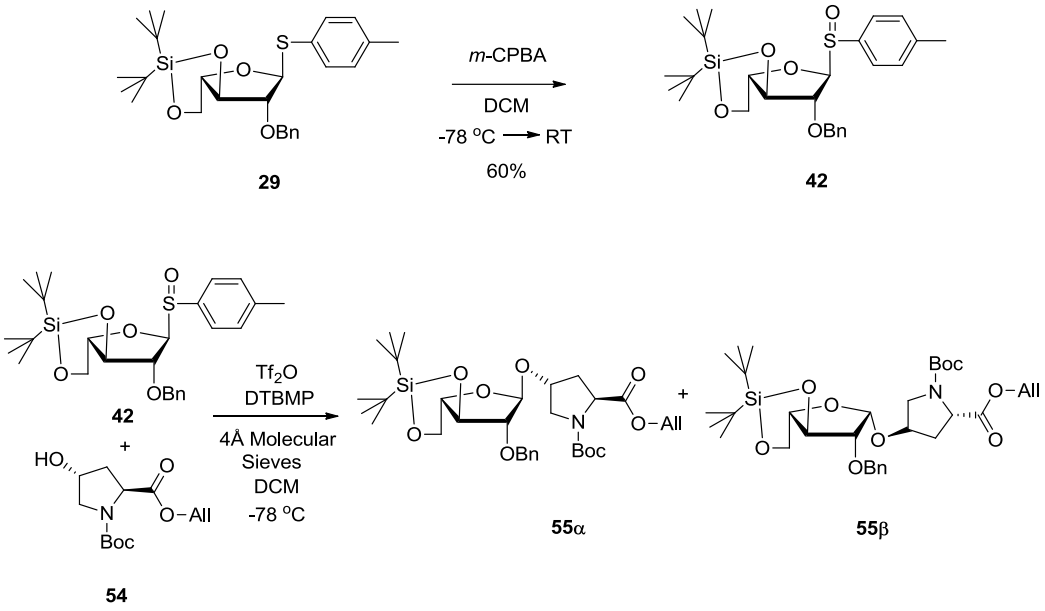
The two singlets at 0.99 ppm and 1.07 ppm, with a total of 18 Hs, can be assigned to the protons of the two *tert*-butyl groups of the silyl acetal. The resonances 27.1 ppm and 27.5 ppm in the ^{13}C spectra can be assigned to the primary carbons of the *tert*-butyl group, while the quaternary carbons are at 20.1 ppm and 22.6 ppm.

Table 2.3 Chemical Shifts for β-Glycoside **55β**.

L -Ara	^1H ppm	^{13}C ppm
H1	4.97 (4.93)	99.2 (100.0)
H2	3.90	80.7
H3	4.31	78.4
H4	3.59	73.6 (73.4)
H5	3.88, 4.31	68.4
CH_2Ph	4.69-4.80	71.8 (71.9)
Si^{t}Bu$_2$	0.99, 1.07	20.1 (4°), 22.6 (4°), 27.1 (1°), 27.5 (1°)
CH_2**Ph**	7.29-7.40	127.7, 127.8, 127.9, 128.0, 128.4, 137.7 (4°)
Hyp		
Hα	4.36-4.44	58.1 (57.7)
Hβ	2.08-2.15, 2.35-2.42 (2.43-2.49)	37.6 (36.8)
Hγ	4.30	75.2
Hδ	3.64	51.1 (51.6)
Boc (*tert*-butyl)	1.38 (1.45)	80.2 (80.1) (4°) 28.2 (28.4) (1°)
COOR	-	153.7 (154.2), 172.7 (172.4)
CH_2CH=CH_2	4.55-4.69	65.5 (65.6)
CH_2**CH**=CH_2	5.85-5.96	131.7 (131.9)
CH_2CH=**CH_2**	5.21-5.35	118.7 (118.3)

*Values in parentheses relate to the minor rotamer about C(=O)N bond of the carbamate.

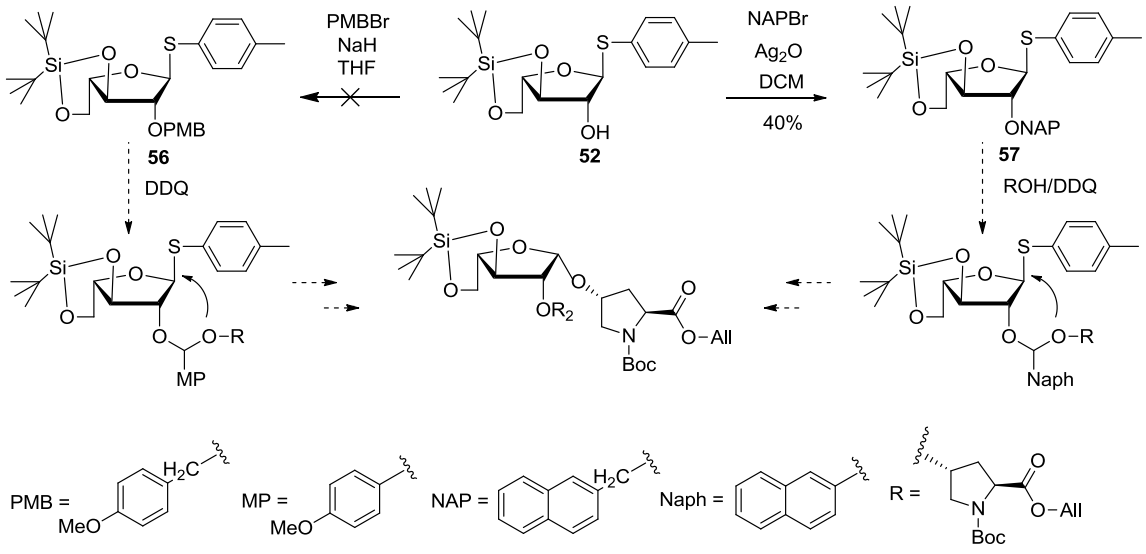
Crich's glycosylation of cyclohexanol with a sulfoxide donor (Scheme 2.10, **42**) gave an average yield but great diastereoselectivity. With the Taylor Group's past experience with sulfoxide donors,[38] this was the next logical approach. Developed by Kahne and coworkers in 1989,[59] the sulfoxide method has proven useful in the glycosylation of sterically hindered nucleophiles. The sulfoxide donor **42** was made by oxidation of the sulfide donor **29** with *m*-CPBA, after which we were able to separate the pair of diastereomers (Scheme 2.17). Unfortunately, the same problems that plagued the sulfide glycosylation still existed, and we were not able to acquire better yields or selectivity under these conditions.



Scheme 2.17 Glycosylation of hydroxyproline with sulfoxide donor

We also briefly investigated a method that would give absolute β-selectivity in glycosylation. Intramolecular aglycon delivery (IAD) allows for the tethering of the nucleophile to the *O*-2 substitutent in a process that would only allow for the formation of a 1,2-*cis*-glycoside. As described in Section 2.3, Prandi published a paper in 2000 that utilizes this method for the construction of β-arabinofuranosides that used a *para*-methoxybenzyl ether as the tethering agent (Scheme 2.18, *vide supra*).[45a] Efforts to synthesize **56** with *para*-methoxybenzyl bromide

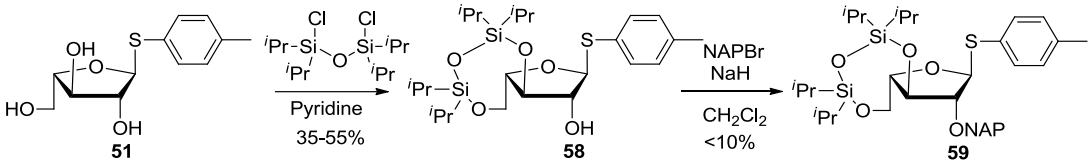
and sodium hydride in THF were unsuccessful due to silyl acetal cleavage. A recent publication by the Ito group gave us another avenue to explore the concept of IAD through the formation of a 2-napthyl ether.[60] Compound **57** has been synthesized in our lab in modest yield. The bulkiness of the naphthylmethyl group makes for a difficult nucleophilic attack by the secondary alcohol. We had synthesized enough of compound **57** that it was possible for us to move on to the next step of tethering the hydroxyproline nucleophile with DDQ, although the instability of the silyl acetal functionality concerned us relating to future reactions.



Scheme 2.18 Intramolecular aglycon delivery strategies

Ito and coworkers have shown that a 3,5-*O*-tetra-*i*-propyldisiloxanylidene protection was optimal in giving β-selectivity when applied to arabinofuranosides.[61] This method, much like the silyl acetal employed by Boons and Crich, used the conformational restriction of the eight membered disiloxane ring to promote the synthesis of the β-anomer. We decided to try this method in the hope that the eight membered disiloxane ring would be more robust under both basic and glycosylating conditions. Treatment of thioglycoside triol **51** in amine base and 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane gave the bicyclic donor **58** in modest yield (Scheme 2.19). Much to our dismay, the eight membered disiloxane ring was no more stable than the

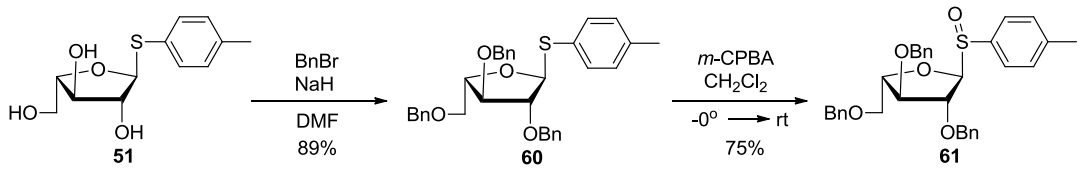
six-membered silyl acetal ring. Subsequent exposure to sodium hydride again showed the cleavage of the 3,5-*O*-protecting group and compound **59** was obtained in unsatisfactory yield.



Scheme 2.19 Preparation of disiloxane donor

2.4.2 Conformationally unrestrained donor

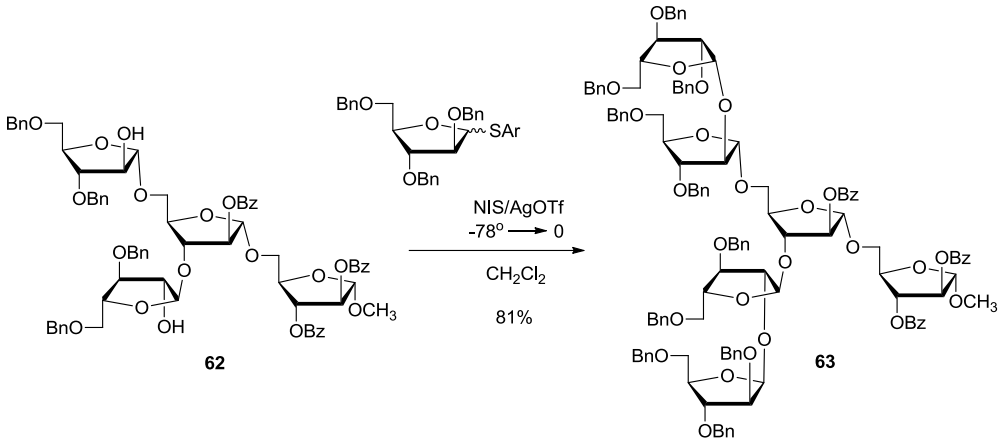
We decided to test a fully benzylated thioglycoside donor (compound **60**, Scheme 2.20) to confirm our suspicion that the labile silyl protecting groups were the cause of our low yields. Compound **51** was transformed into the tri-*O*-benzyl arabinofuranothioglycoside **60** using standard methods (benzyl bromide, NaH, DMF) in great yield. The success of this benzylation reaction reinforced our hypothesis about the shortcomings of the 3,5-*O*-silyl protecting groups. Just to be thorough, we also made the corresponding sulfoxide **61** in good yield.



Scheme 2.20 Preparation of two conformationally unrestrained donors

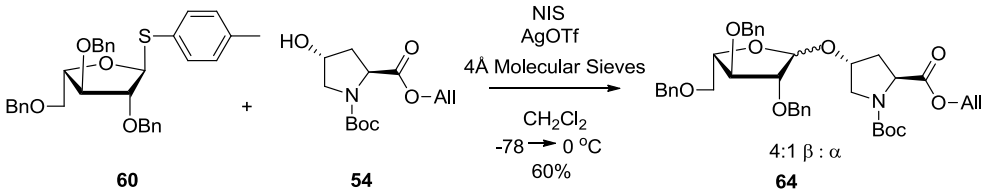
Lowary and coworkers, in their synthesis of a naturally-occurring, highly branched arabinofuranosyl hexasaccharide, had reported a surprising highly stereoselective synthesis of β-arabinofuranosides.[62] Using *N*-iodosuccinimide and silver triflate as activator, the hexasaccharide **63** was obtained in high yield and with 100% β-selectivity (Scheme 2.21). The authors reported that careful control of temperature is critical as an increase in side products

was observed when the reaction was run at -40 or 0 ^{o}C. We employed this procedure in the hope of finding similar success for our purpose.



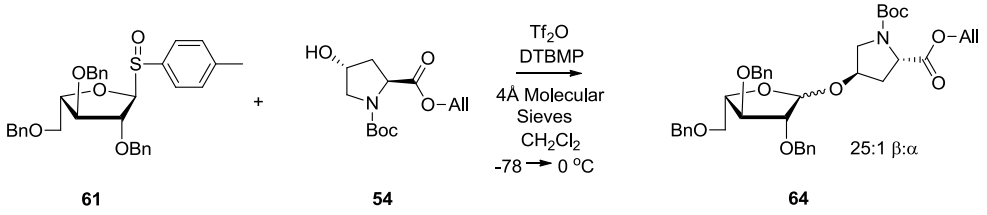
Scheme 2.21 Low temperature glycoysylation method

To our delight, reaction of thioglycoside **60** with our hydroxyproline acceptor gave a reasonable yield (60%) with an average β:α ratio of 4:1 (Scheme 2.22), a significant improvement relative to Scheme 2.16 in both aspects. Also important is the fact that we are able to separate the α- and β-anomers by means of flash chromatography, an added bonus that would save us much time and grief as compared to HPLC. Careful upscaling of this reaction has allowed us to do the glycosylation on a multigram level, and we are able to obtain spectroscopically pure β-glycoside up to a gram at a time. Further advantages of this route include saving one linear step in the overall synthesis while also substituting for two reactions that gave typical yields of ~35% (silyl acetal protection, benzyl ether protection) with a single protection step that yields up to 90% (benzylation). The tri-*O*-benzylated arabinofuranosides are robust and can withstand a variety of acidic and basic conditions. Down the line, the perbenzylated arabinosides can be deprotected by a single hydrogenolytic step to remove all benzyl ethers.



Scheme 2.22 Glycosylation of hydroxyproline with benzylated sulfide donor

We also applied the sulfoxide method to making our β-arabinosides of hydroxyproline (Scheme 2.23). The sulfoxide donor **61** is activated by triflic anhydride and a sterically hindered amine base acts as a buffer for the reaction. The sulfoxide glycosylation gave similar yields but offered far superior selectivity (25:1 β:α by integration of anomeric proton) than its sulfide counterpart. However, the reaction was plagued by side products that are indistinguishable chromatographically from our wanted compounds. Moreover, the stability of the triflic anhydride, even when left unopened, is a deterrent for this reaction compared to the relatively stable NIS/AgOTf. This practical application, in combination with the extra step required to form the sulfoxide, led us to favor the sulfide donor **60** for the large scale preparation of the β-monomer. β-Glycoside **64β** has been fully characterized by mass spectromery, ^{1}H, ^{13}C, and 2-D NMR spectra.

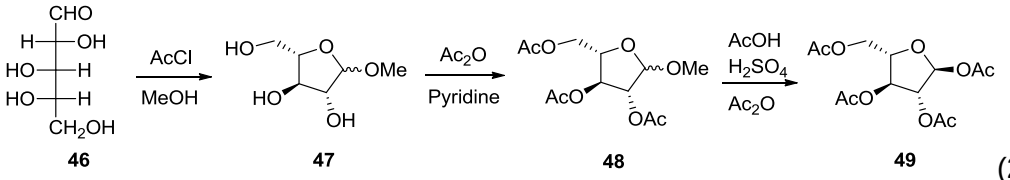


Scheme 2.23 Glycosylation of hydroxyproline with benzylated sulfoxide donor

2.5 Experimental Section

General methods: All reactions were performed under a dry nitrogen atmosphere unless otherwise noted. Reagents were obtained from commercial sources and used directly; exceptions are noted. Diisopropylethylamine, triethylamine, and pyridine were dried and distilled from CaH_2 and stored over KOH pellets. Ethanol and methanol were distilled from Mg turnings and stored over 3 Á molecular sieves. Flash chromatography was performed using flash silica gel (32-63 µ) from Dynamic Adsorbents Inc. Reactions were followed by TLC on precoated silica plates (200 µm, F-254 from Dynamic Adsorbents Inc.). The compounds were visualized by UV fluorescence or by staining with ninhydrin, $KMnO_4$, or 10% sulfuric acid in ethanol stains. NMR spectra were recorded on Bruker DPX-250, AV-400-liquid, or Varian 700 MHz spectrometers. Proton NMR data is reported in ppm downfield from TMS as an internal standard. Disodium 3-trimethylsilyl-1-propane-sulfonate (DSS) was used to reference ^1H NMR spectra run in D_2O. High resolution mass spectra were recorded using either time-of-flight or electrospray ionization.

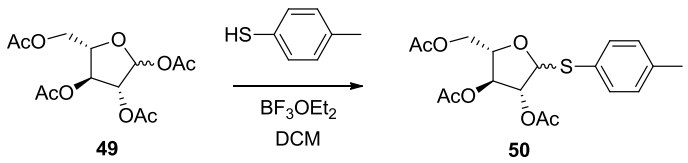
2.5.1 Experimental procedures



46 **47** **48** **49** (2.1)

Peracylated-α-L-*Arabinoside* (**49**). *L*-Arabinose (5.0 g, 33.3 mmol, 1 equiv.) was dissolved in methanol (100 mL) and treated with a solution of acetyl chloride (2.5 mL) in methanol (30 mL) at rt under N_2. The mixture was stirred for 3 h, during which time the solid completely dissolved. The reaction was quenched dropwise with pyridine. The solvent was evaporated, followed by azeotroping with DCM to give the methyl arabinoside as a mixture of anomers. The crude product was dissolved in pyridine (40 mL) and cooled to 0 °C, after which acetic anhydride (20

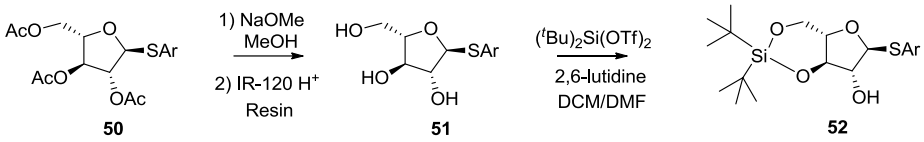
mL) was added and the reaction was stirred overnight at rt. The solvent was evaporated and the mixture was diluted with CH_2Cl_2 (250 mL), washed with water (250 mL), 1 M HCl (250 mL), sat. $NaHCO_3$ (250 mL), and brine (250 mL). The organic phase was filtered through $MgSO_4$ and concentrated to give the triacetate. The crude product was dissolved in acetic anhydride (80 mL) and cooled to 0 °C. Acetic acid (20 mL) was added dropwise. After 15 min, sulfuric acid (5 mL) was added dropwise. The mixture was warmed to rt while stirring for approximately 2 h. The solution was poured over a mixture of ice (50 g), CH_2Cl_2 (250 mL) and sat'd aq. $NaHCO_3$ (200 mL). The organic layer was separated and washed again with several volumes of sat'd aq. $NaHCO_3$, filtered through $MgSO_4$, and concentrated to give the crude peracylated furanoside as a light oil (9.071 g, 85%; 3 steps).



49 **50** (2.2)

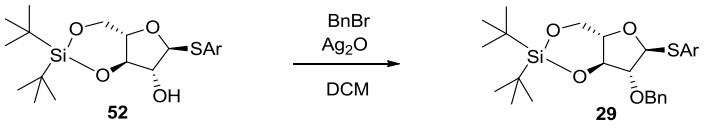
p-Cresyl 2,3,5-O-acetyl-1-thio-α-L-arabinofuranoside (**50**). A solution of **49** (4.47 g, 14 mmol, 1.0 equiv.) and *p*-thiocresol (2.64 g, 21 mmol, 1.5 equiv.) in dry CH_2Cl_2 (60 mL) was cooled to 0 °C under N_2. Boron trifluoride diethyl etherate (1.0 mL, 1.17 g, 8.1 mmol, 0.5 equiv.) was added dropwise and the mixture stirred for 5 h under N_2 at 0 °C. The reaction was quenched with Et_3N (4 mL) and concentrated. The mixture was diluted with EtOAc (150 mL) and washed with H_2O (150 mL) and brine (150 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash chromatography eluting with 3:1 Hex/EtOAc to give **50** as a light colored oil (3.54 g, 66 %). R_f 0.25 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ -170.2 (*c* = 1, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.10 (s, 3H), 2.11 (s 3H), 2.13 (s, 3H), 2.34 (s, 3H), 4.28 (dd, *J* = 12.1, 5.5 Hz, 1H), 4.39 (dd, *J* = 12.1, 3.6 Hz, 1H), 4.48 (app. q, *J* = 4.7 Hz, 1H), 5.07 (d, *J* = 5.5 Hz, 1H), 5.27 (s, 1H), 5.47 (s, 1H), 7.13 (d, *J* = 7.8 Hz, 2H), 7.40 (d, *J* = 7.8 Hz, 2H); ^{13}C NMR (100

MHz, $CDCl_3$) δ 20.7 (3C), 21.1, 62.8, 77.2, 79.9, 81.4, 91.2, 129.6, 129.8, 132.7, 138.1, 169.6, 170, 170.5; HRMS (ESI) calcd for $C_{18}H_{21}O_7SNa$ $(M+Na)^+$: 405.0978; obsd: 405.0985.



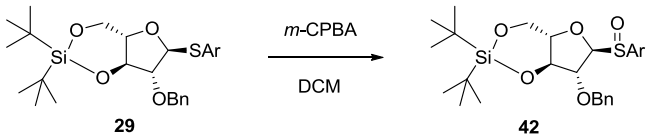
(2.3)

p-Cresyl 3,5-O-(Di-tert-*butylsilylene)-1-thio-α-L-arabinofuranoside* (**52)**. Thioglycoside triacetate **50** (694 mg, 1.8 mmol) was dissolved in MeOH (15 mL). Sodium methoxide (200 μL, 25% in MeOH) was added and stirred under N_2 overnight. Amberlite® IR-120 acid resin was added portionwise while stirring until solution was neutralized, after which it was filtered and rinsed with MeOH, and concentrated. The crude triol **51** (363 mg, 1.4 mmol, 1.0 equiv.) was suspended in a mixture of dry CH_2Cl_2 (13 mL) and DMF (2.5 mL) and cooled to 0 °C under N_2. 2,6-Lutidine (762 μL, 705 mg, 6.6 mmol, 4.7 equiv.) and di-*tert*-butylsilyl *bis*-(trifluoromethanesulfonate) (545 μL, 741 mg, 1.7 mmol, 1.2 equiv.) were then added sequentially. The mixture was stirred overnight under N_2 at room temperature. The mixture was concentrated and the residue diluted with EtOAc (30 mL) and washed with H_2O (30 mL) and brine (30 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash chromatography eluting with 15:1 Hex/EtOAc to give **52** as an amorphous colorless solid (297 mg, 53%). R_f 0.49 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ -181.0 (*c* = 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.00 (s, 9H), 1.08 (s, 9H), 2.33 (s, 3H), 3.58 (d, *J* = 4.2 Hz, 1H), 3.87-3.98 (m, 2H), 4.02 (app. t, *J* = 8.3 Hz, 1H), 4.14 (app. q, *J* ~ 5 Hz, 1H), 4.35 (dd, *J* = 8.3, 4.2 Hz, 1H), 5.27 (d, *J* = 5.9 Hz, 1H), 7.12 (d, *J* = 7.8 Hz, 2H), 7.42 (d, *J* = 7.9 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.1, 21.1, 22.7, 27.1, 27.5, 67.4, 73.7, 80.6, 77.4, 91.5, 129.8, 130.3, 132.3, 137.9; HRMS (ESI) calcd for $C_{20}H_{33}O_4SSi$ $(M+H)^+$: 397.1863; obsd: 397.1856.



(2.4)

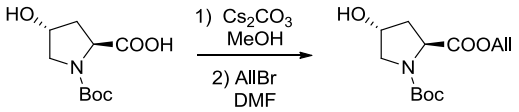
p-Cresyl 2-O-Benzyl-3,5-O-(di-tert-*butylsilylene)-1-thio-α-L-arabinofuranoside* (**29**). Compound **52** (400 mg, 1.1 mmol, 1 equiv.) was dissolved in dry $CHCl_2$ (11 mL) and stirred under N_2. Benzyl bromide (240 μL, 345 mg, 2.0 mmol, 2 equiv.) was added, followed by Ag_2O (714 mg, 3.1 mmol, 3 equiv.). The mixture was stirred for 3 d, filtered through an inch of silica and washed with $CHCl_2$, and concentrated. The residue was purified by flash chromatography eluting with 120:1 Hex/Ether → 60:1 Hex/EtOAc to give **29** as a colorless solid (336 mg, 64%). R_f 0.62 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ -129.93 (*c* = 1, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.97 (s, 9H), 1.06 (s, 9H), 2.28 (s, 3H), 3.84-3.98 (m, 3H), 4.12 (app. t, *J* = 8.4 Hz, 1H), 4.31 (dd, *J* = 8.2, 4.1 Hz, 1H), 4.72 (d, *J* = 12.0 Hz, 1H), 4.81 (d, *J* = 12.0 Hz, 1H), 5.34 (d, *J* = 5.2 Hz, 1H), 7.05 (d, *J* = 7.9 Hz, 2H), 7.26-7.40 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.2, 21.2, 22.7, 27.2, 27.6, 67.4, 72.2, 73.8, 81.4, 86.8, 90.3, 127.9, 128.1, 128.5, 129.8, 130.6, 132.3, 137.71, 137.74; HRMS (ESI) calcd for $C_{27}H_{39}O_4SSi$ $(M+H)^+$: 487.2333; obsd: 487.2324.



(2.5)

p-Cresyl 2-O-Benzyl-3,5-O-(di-tert-*butylsilylene)-1-thio-α-L-arabinofuranoside S-Oxide* (**42**). A solution of 3-chloroperoxybenzoic acid (63.5 mg, 77 wt %, 2.8 mmol, ~1.2 equiv.) in dry CH_2Cl_2 (1 mL) was added dropwise to a solution of compound **29** (116 mg, 0.24 mmol, 1 equiv.) in dry CH_2Cl_2 (4 mL) at -80 °C under Argon and stirred. The reaction mixture was gradually warmed to room temperature over 2 h. The solution was diluted with CH_2Cl_2 (30 mL), washed with sat'd aq $NaHCO_3$ (30 mL), filtered through $MgSO_4$, and concentrated. The residue was purified by column chromatography, eluting with 10:1 Hex/EtOAc to afford **42** as a colorless gel (68 mg,

57%) and a mixture of diastereomers varying in their configuration at sulfur. Diastereomer A: R_f 0.42 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ 101.4 (*c* = 1, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 1.00 (s, 9H), 1.05 (s, 9H), 2.40 (s, 3H), 3.89 (app. t, *J* = ~10 Hz, 1H), 3.99-4.05 (td, J = 10, 5.0 Hz, 1H), 4.22 (dd, *J* = ~10, 7.2 Hz, 1H), 4.30 (d, *J* = 11.4 Hz, 1H), 4.35 (dd, *J* = 9.0, 5.0 Hz, 1H), 4.57-4.59 (m, 1H), 4.58 (d, *J* = 11.4 Hz, 1H), 4.65 (d, *J* = 5.1 Hz, 1H), 6.98 (app. t, *J* = 3.6 Hz, 2H), 7.25-7.27 (m, 3H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.2, 21.4, 22.6, 27.1, 27.4, 67.4, 72.0, 77.0, 78.5, 82.0, 99.3, 124.4, 127.7, 127.9, 128.2, 129.9, 136.2, 137.3, 141.7; HRMS (ESI) calcd for $C_{27}H_{39}O_5SSi$ $(M+H)^+$: 503.2282; obsd: 503.2298; Diastereomer B: R_f 0.33 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ -178.6 (*c* = 1.35, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) δ 0.95 (s, 9H), 1.05 (s, 9H), 2.40 (s, 3H), 3.78-3.84 (m, 1H), 3.86 (app. t, *J* = ~9.4 Hz, 1H), 4.20 (app. t, *J* = 8.2 Hz, 1H), 4.30 (dd, *J* = 8.6, 4.5 Hz, 1H), 4.35 (app. t, *J* = 6.4 Hz, 1H), 4.58 (d, *J* = 11.7 Hz, 1H), 4.63 (d, *J* = 5.6 Hz, 1H), 4.76 (d, *J* = 11.7 Hz, 1H), 7.23-7.36 (app. m, 7H), 7.52 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.0, 21.5, 22.6, 27.0, 27.4, 67.3, 72.3, 76.9, 81.1, 81.5, 98.2, 125.6, 128.0, 128.1, 128.4, 129.9, 136.4, 137.3, 142.3; HRMS (ESI) calcd for $C_{27}H_{39}O_5SSi$ $(M+H)^+$: 503.2282; obsd: 503.2294.

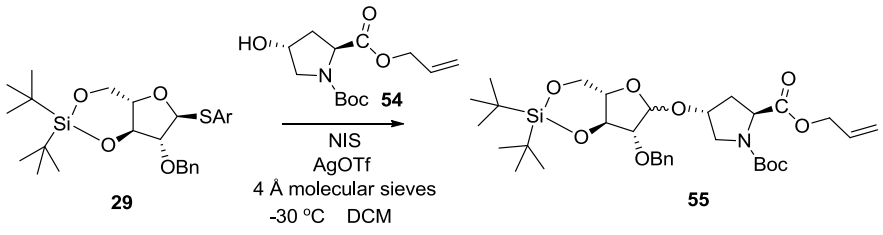


53 **54** (2.6)

N-tert-*Butoxycarbonyl*-trans-*4-hydroxy*-L-*proline allyl ester* (**54**). To a suspension of Boc-Hyp-OH (**53**) (1.77 g, 7.7 mmol, 1.0 equiv.) in dry MeOH (16 mL) was added cesium carbonate (1.37 g, 4.2 mmol, 0.55 equiv.). The mixture was stirred under N_2 for 1.5 h during which time the reaction mixture became a homogeneous solution. The solvent was evaporated, and the residue dissolved in dry DMF (10 mL) and treated immediately with allyl bromide (1.2 g, 0.86 mL, 9.9 mmol, 1.3 equiv.). The mixture was stirred overnight at RT under N_2. The mixture was diluted with EtOAc (150 mL) and washed with H_2O (150 mL) and brine (150 mL). The organic

layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 2:1 EtOAc/Hex to give the ester **54** as a light oil (1.86 g, 89%). R_f 0.34 (2:1 EtOAc/Hex). $[\alpha]_D^{25}$ -65.0 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (1.46)* (s, 9H), 2.03-2.10 (m, 1H), 2.25-2.35 (m, 1H), 2.96 (s, 1H), 3.44-3.65 (m, 2H), 4.40-4.48 (m, 2H), 4.56-4.71 (m, 2H), 5.25 (app. t, *J* = ~11.3 Hz, 1H), 5.35 (dt, *J* = 17.1, 3.8, 1.2 Hz, 1H), 5.87-5.95 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.2 (28.3), 39.1 (38.3), 54.6, 58.0 (57.7), 65.6, 69.1 (69.8), 80.5 (80.2), 118.8 (118.3), 131.6 (131.8), 154.1 (154.6), 172.9 (172.6); HRMS (ESI) calcd for $C_{13}H_{21}NO_5Na$ $(M+Na)^+$: 294.1312, obsd: 294.1320.

* values in parentheses signifiy a second signal due to a minor rotamer

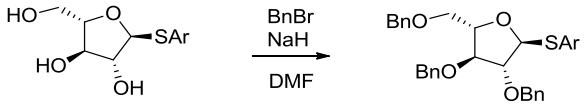


(2.7)

Nα-tert-*Butyloxycarbonyl*-trans-*4-hydroxy-4-O-[2-O-Benzyl-3,5-O-(di*-tert-*butylsilylene)-L-arabinofuranosyl]-L-proline Allyl Ester* **55**. A solution of compounds **29** (219 mg, 0.45 mmol, 1.0 equiv.) and **54** (250 mg, 0.92 mmol, 2.0 equiv.) in dry CH_2Cl_2 (30 mL) were added to a flask containing activated 4 Å crushed molecular sieves under N_2. The suspension was stirred for ~20 min at RT then cooled to -65 °C (ethylene glycol and dry ice). The temperature dropped to -30 °C and NIS (173 mg, 0.68 mmol, 1.5 equiv.) was added, followed by a solution of AgOTf (57 mg, 0.22 mmol, 0.5 equiv) in toluene (0.6 mL) as the temperature continued to drop to -65 °C. The suspension was stirred for 1 hr during which it was allowed to gradually warm to RT. Solution continued to stir until room temperature was reached. After ~20 mins at room temperature, the reaction was quenched with Et_3N, filtered, and concentrated. The residue was diluted with EtOAc (50 mL) and washed with sat'd aq. $Na_2S_2O_3$ (50 mL) and brine (50 mL),

filtered through $MgSO_4$, and concentrated. The residue was purified by column chromatography, eluting with 10:1 Hex/EtOAc to afford crude **55** as a colorless oil (147 mg, 51%) as a mixture of anomers and rotamers. Crude compound **55** was subjected to HPLC, eluting with 20% EtOAc in Hexanes at 13.0 mL min^{-1} on a 21 mm silica column detecting with UV at 254 nm. R_T (α) = 13 min, R_T (β) = 23 min; β-anomer: R_f 0.30 (3:1 Hexanes/EtOAc). ^1H NMR (400 MHz, $CDCl_3$) δ 0.99 (s, 9H), 1.07 (s, 9H), 1.38 (1.45)* (1 s, 9H), 2.08-2.15 (m, 1H), 2.35-2.42 (2.43-2.49) (m, 1H), 3.58-3.69 (m, 3H), 3.86-3.91 (m, 2H), 4.26-4.32 (m, 3H), 4.36-4.44 (m, 1H), 4.56-4.73 (m, 3H), 4.79 (d, J = 12.2 Hz, 1H), 4.97 (4.93) (d, J = ~5.3 Hz, 1H), 5.24 (app. t, J = 11.6 Hz, 1H), 5.32 (ddd, J = 17.2, 5.9, 1.3 Hz, 1H), 5.85-5.96 (m, 1H), 7.29-7.40 (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.1, 22.6, 27.1, 27.5, 28.2 (28.4), 37.6 (36.8), 51.1 (51.6), 58.1 (57.7), 65.5 (65.6), 68.4, 71.8 (71.9), 73.6 (73.4), 75.2, 78.4, 80.2 (80.1), 80.7, 99.2 (100.0), 118.7 (118.3), 127.7, 127.8, 127.9, 128.0, 128.4, 131.7 (131.9), 137.7, 153.7 (154.2), 172.7 (172.4); HRMS (ESI) calcd for $C_{33}H_{52}NO_9Si$ $(M+H)^+$: 634.3406; obsd: 634.3408.

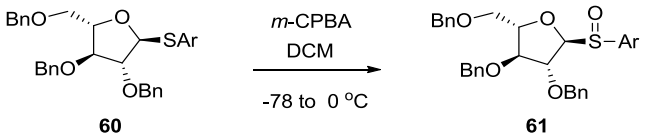
* values in parentheses signifiy a second signal due to a minor rotamer



51 **60** (2.8)

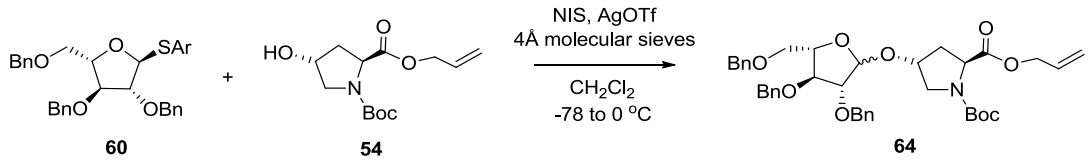
p-Cresyl 2,3,5-O-Benzyl-1-thio-α-L-arabinofuranoside (**60**). Triol **51** (1.91 g, 7.5 mmol, 1.0 equiv.) was dissolved in dry DMF (15 mL) and cooled to 0 °C under N_2. Benzyl bromide (5.3 mL, 7.61 g, 44.5 mmol, 6.0 equiv.) and NaH (1.78 g, 60% dispersion, 44.5 mmol, 6.0 equiv.) were added sequentially. The reaction mixture was allowed to warm to RT while it stirred under N_2 for 3 h. The reaction was quenched with sat'd $NaHCO_3$ (150 mL) and extracted with CH_2Cl_2 (150 mL). The organic layer was washed with H_2O (150 mL) and brine (150 mL), filtered through $MgSO_4$, and concentrated. The residue was purified by flash chromatography eluting with 20:1 → 10:1 → 1:2 Hex/EtOAc to give **60** as a clear gel (3.26 g, 84%; 2 steps). R_f 0.46 (3:1

Hexanes/EtOAc). $[\alpha]_D^{25}$ -107.9 (*c* = 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.32 (s, 3H), 3.63 (dd, *J* = 10.8, 4.7 Hz, 1H), 3.68 (dd, *J* = 10.8, 3.9 Hz, 1H), 4.03 (app. q, *J* = 3.3 Hz, 1H), 4.11 (t, *J* = 2.9 Hz, 1H), 4.36-4.38 (m, 1H), 4.48-4.65 (m, 6H), 5.53 (d, *J* = 2.0 Hz, 1H), 7.10 (d, *J* = 7.8 Hz, 2H), 7.26-7.35 (m, 15H), 7.41 (d, *J* = 7.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.2, 69.1, 72.7, 72.3, 73.4, 80.5, 83.5, 88.4, 90.6, 127.6, 127.7, 127.8, 127.9, 127.9, 128.0, 128.3, 128.4, 128.5, 129.7, 131.0, 132.0, 137.4, 137.8, 138.2; HRMS (ESI) calcd for $C_{33}H_{34}NaO_4S$ $(M+Na)^+$: 549.2070; obsd: 549.2067.



60 **61** (2.9)

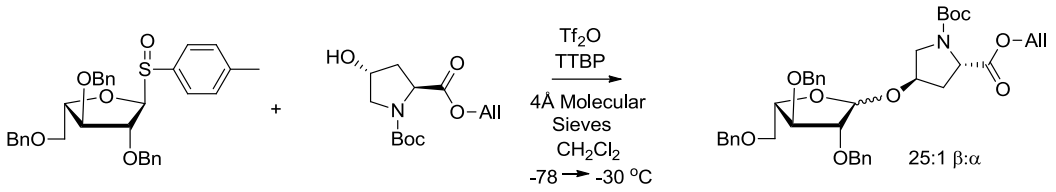
p-Cresyl 2,3,5-O-Benzyl-1-thio-α-L-arabinofuranoside S-Oxide **61**. A solution of 3-chloroperoxybenzoic acid (283 mg, 77 wt %, 1.3 mmol, 1.2 equiv.) in dry CH_2Cl_2 (4 mL) was added dropwise to a solution of compound **60** (555 mg, 1.1 mmol, 1.0 equiv.) in dry CH_2Cl_2 (16 mL) at -78 °C under N_2 and stirred. The reaction mixture was gradually warmed to RT over 1.5 h. The solution was diluted with CH_2Cl_2 (70 mL), washed with sat'd $NaHCO_3$ (70 mL), filtered through $MgSO_4$, and concentrated. The residue was purified by column chromatography, eluting with 3:1 Hex/EtOAc to afford **61** as a colorless gel (429mg, 75%) and essentially a single diastereomer (uneven mixture in which the amount of minor isomer is negligible). Major diastereomer: R_f 0.19 (3:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ +30.1 (*c* 1.0 , CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.39 (s, 3H), 3.53 (dd, *J* = 10.6, 5.5 Hz, 1H), 3.60 (dd, *J* = 10.6, 4.8 Hz, 1H), 4.15 (dd, *J* = 5.5, 2.8 Hz, 1H), 4.37-4.51 (m, 6H), 4.59 (d, *J* = 11.8 Hz, 1H), 4.63 (d, *J* = 1.8 Hz, 1H), 4.74 (app. t, *J* = ~2.4 Hz, 1H), 7.20-7.32 (m, 17H), 7.57 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.5, 69.1, 71.9, 72.2, 73.3, 83.3, 83.9, 84.4, 102.0, 124.8, 127.7, 127.9, 128.0 (2 signals), 128.4 (2 signals), 128.5, 129.9, 137.3, 137.4, 137.9, 138.7, 141.8; HRMS (ESI) calcd for $C_{33}H_{35}O_5S$ $(M+H)^+$: 543.2160; obsd: 543.2206.



(2.10)

N*α*-tert-*Butyloxycarbonyl*-trans-*4-hydroxy-4-O-[2,3,5-O-Benzyl-L-arabinofuranosyl]-L-proline Allyl Ester* (**64**). A solution of compounds **60** (1.17 g, 2.2 mmol, 1.0 equiv.) and **54** (0.688 g, 2.5 mmol, 1.1 equiv.) in dry CH_2Cl_2 (40 mL) was stirred with activated 4 Å molecular sieves (3.5 g) under N_2 for ~30 min at RT. The suspension was cooled to -78 °C (acetone/dry ice) and then NIS (0.747 g, 3.2 mmol, 1.5 equiv.) and AgOTf (0.285 g, 0.55 mmol, 0.5 equiv.) were added. The reaction was allowed to gradually reach 0 °C over 1.5 h, at which time it was quenched with Et_3N (3 mL) and filtered. The filtrate was diluted with EtOAc (150 mL) and washed with 10% aqueous $Na_2S_2O_3$ (2 x 200 mL) and brine (200 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue, determined to be a 4:1 β:α ratio by NMR, was purified by column chromatography, eluting with 3:1 Hex/EtOAc to afford **64β** (the β-anomer) (0.732 g, 50%, 2:1 ratio of rotamers) as an orange oil. R_f 0.56 (1:1 Hexanes/EtOAc). $[\alpha]_D^{25}$ +17.9 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37 (1.44)* (s, 9H), 1.96-2.07 (m, 1H), 2.27-2.37 (m, 1H), 3.44-3.67 (m, 4H), 4.03-4.12 (m, 3H), 4.25-4.71 (m, 10H), 4.96 (4.92) (d, *J* = 3.2 Hz, 1H), 5.20-5.34 (m, 2H), 5.86-5.91 (m, 1H), 7.28-7.33 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.3 (28.4), 37.4 (36.5), 50.7 (51.4), 58.1 (57.8), 65.6 (65.7), 72.0, 72.4, 73.3, 75.5, 80.1, 80.3, 82.7, 83.8, 83.9, 99.0 (99.9), 118.8 (118.4), 127.6, 127.8, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 128.5; 131.7 (131.9), 137.4, 137.9, 138.1, 153.7 (155.4), 172.7 (172.4). HRMS (ESI) calcd for $C_{39}H_{48}NO_9$ $(M+H)^+$: 674.3324, obsd: 674.3343.

* values in parentheses signifiy a second signal due to a minor rotamer



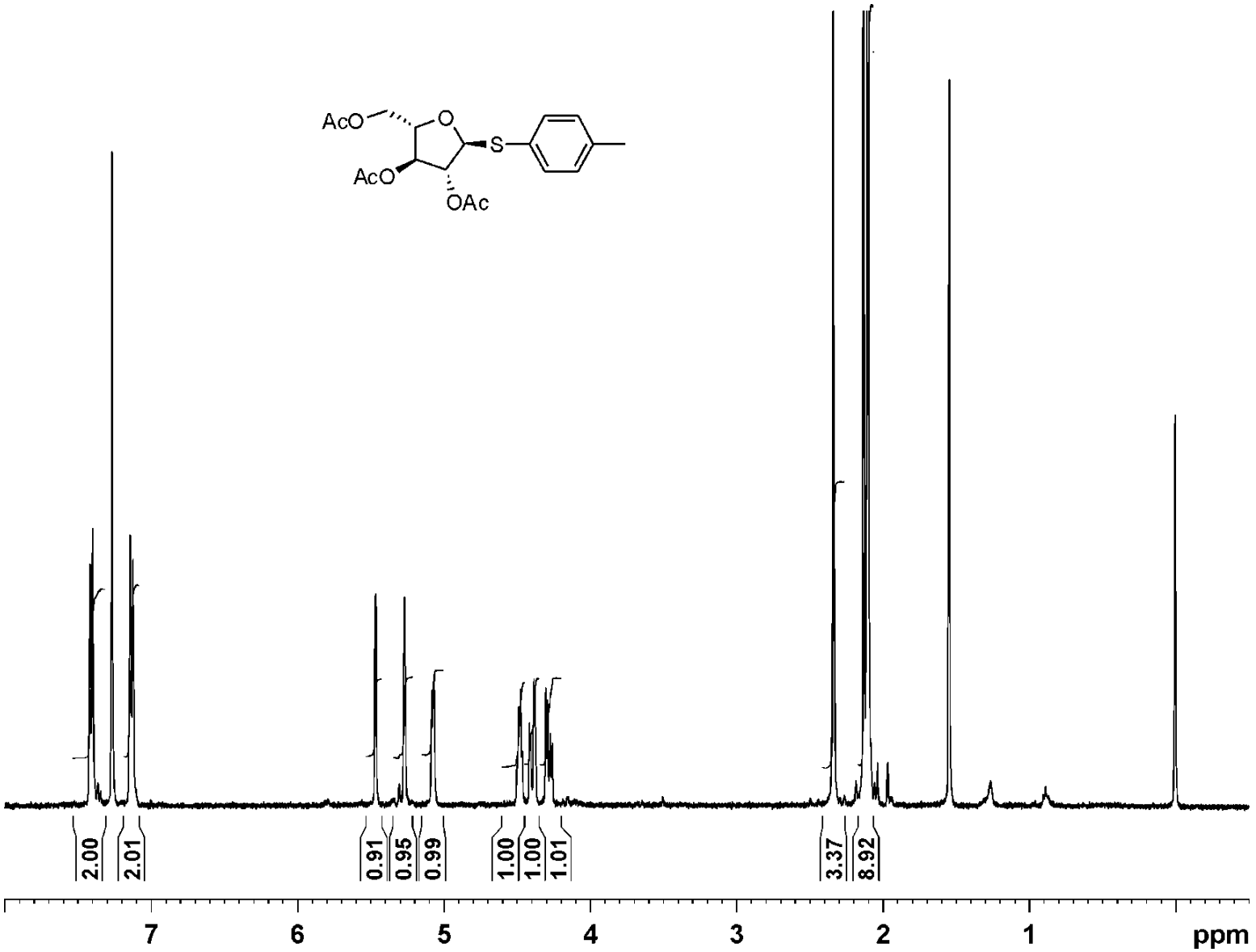
61 **54** **64** (2.11)

N*α*-tert-*Butyloxycarbonyl*-trans*-4-hydroxy-4-O-[2,3,5-O-Benzyl-L-arabinofuranosyl]-L-proline Allyl Ester* (**64**) – Sulfoxide method. A solution of compound **61** (133 mg, 0.25 mmol, 1.0 equiv.) and 2,4,6-tri-*tert*-butyl pyridine (121 mg, 0.49 mmol, 2 equiv.) (TTBP) in dry CH_2Cl_2 (5 mL) was stirred with activated 4Å molecular sieves (500 mg) under N_2 for ~30 min at RT. The suspension was cooled to -78 °C (acetone/dry ice) and then triflic anhydride (49 µL, 82 mg, 0.29 mmol, 1.2 equiv.) was added, followed by compound **54** (66 mg, 0.24 mmol, 1.0 equiv.). The reaction was allowed to gradually reach -30 °C over 2 h, at which time it was quenched with Et_3N (3 mL), filtered, and concentrated. The residue, determined to contain a 25:1 β:α ratio of **64** by NMR, was purified by column chromatography, eluting with 3:1 Hex/EtOAc to afford the β-anomer **64β** (84 mg, 51%) as an orange oil.

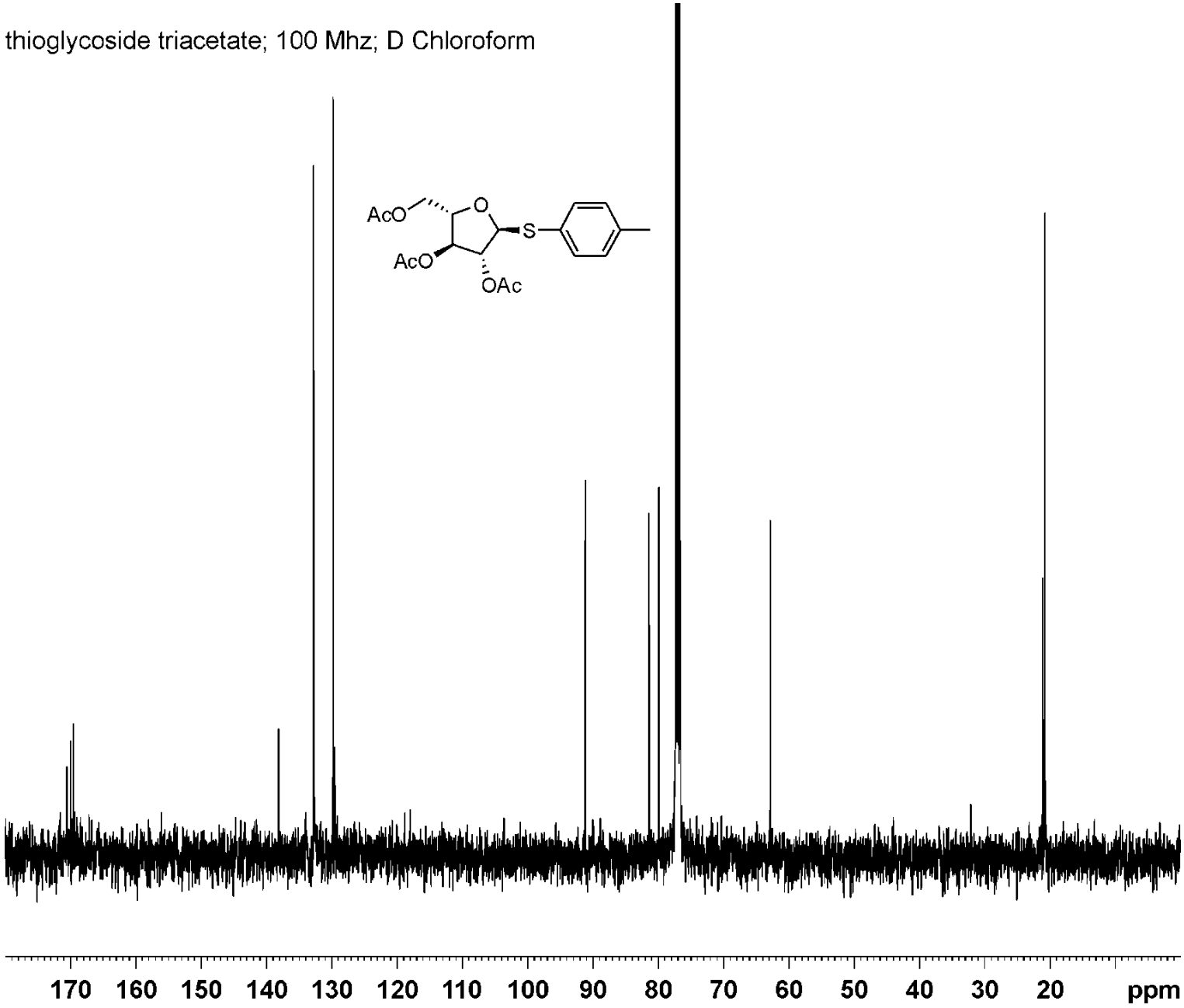
2.5.2 Spectra

Compound **50** (Scheme 2.11) - ^{1}H NMR spectrum

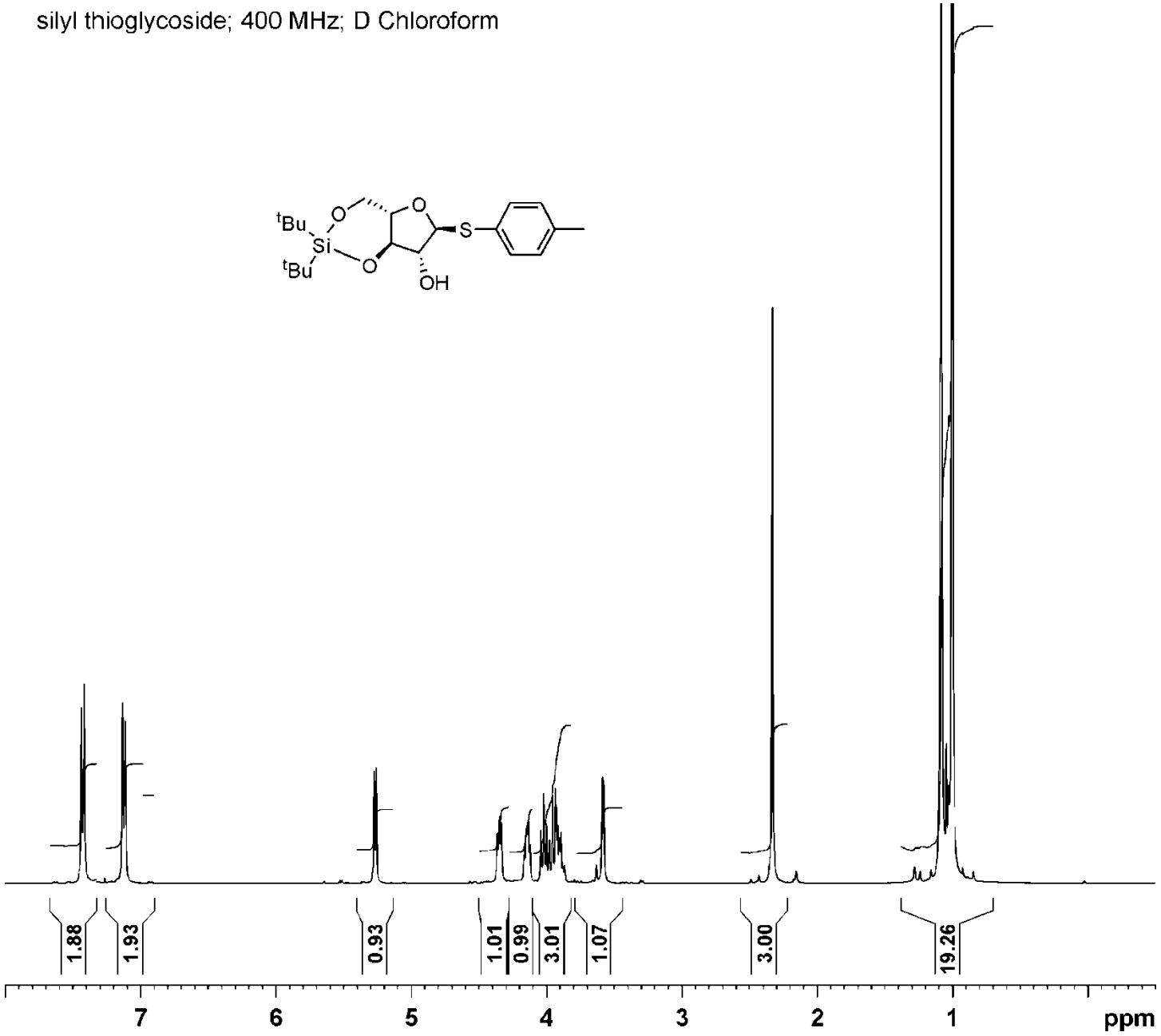
thioglycoside triacetate; 400 Mhz; D Chloroform



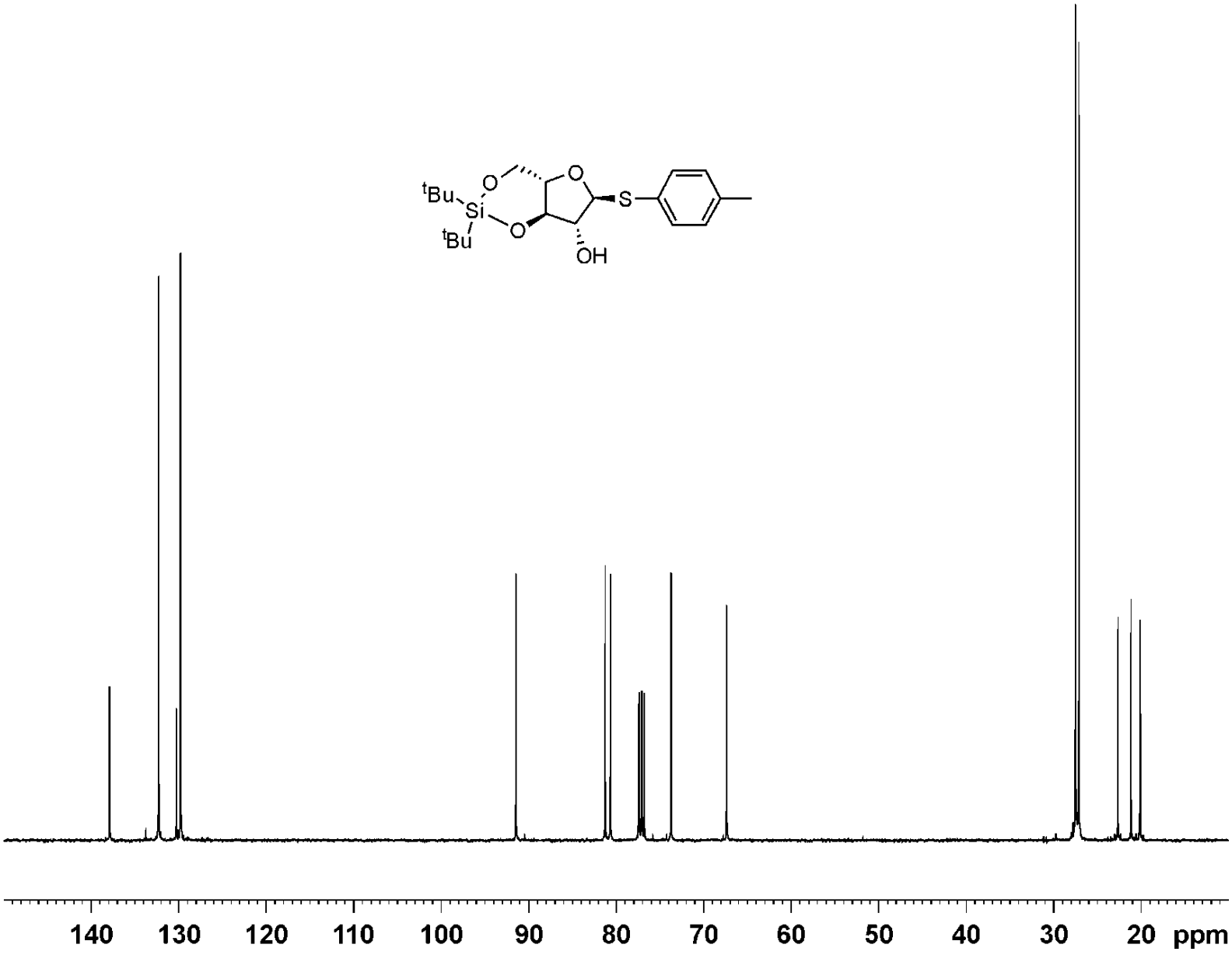
Compound **50** (Scheme 2.11) – ^{13}C NMR spectrum



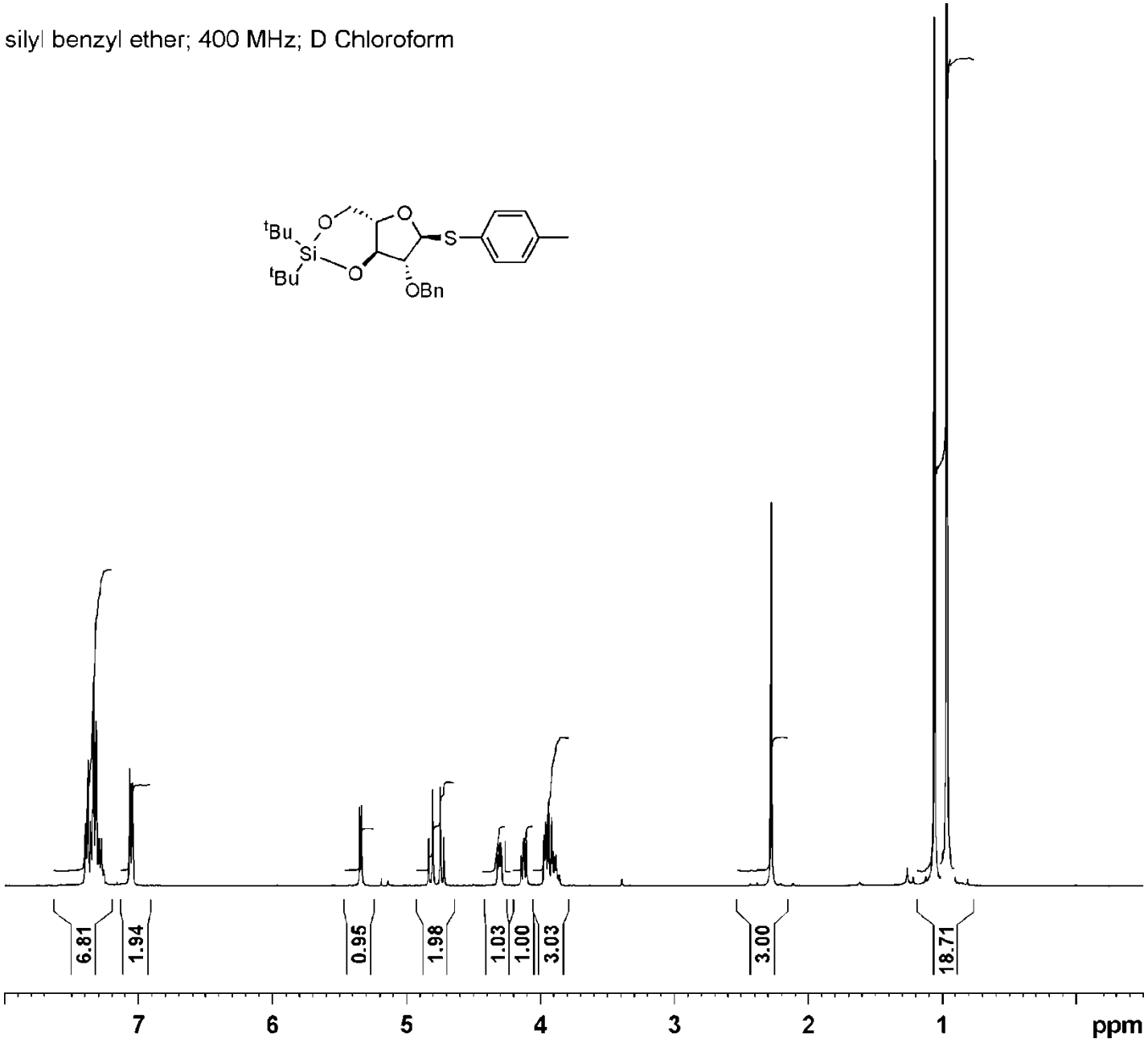
Compound **52** (Scheme 2.11) - ^{1}H NMR spectrum



Compound **52** (Scheme 2.11) – ^{13}C NMR spectrum
silyl acetal thioglycoside; 100 Mhz; D Chloroform

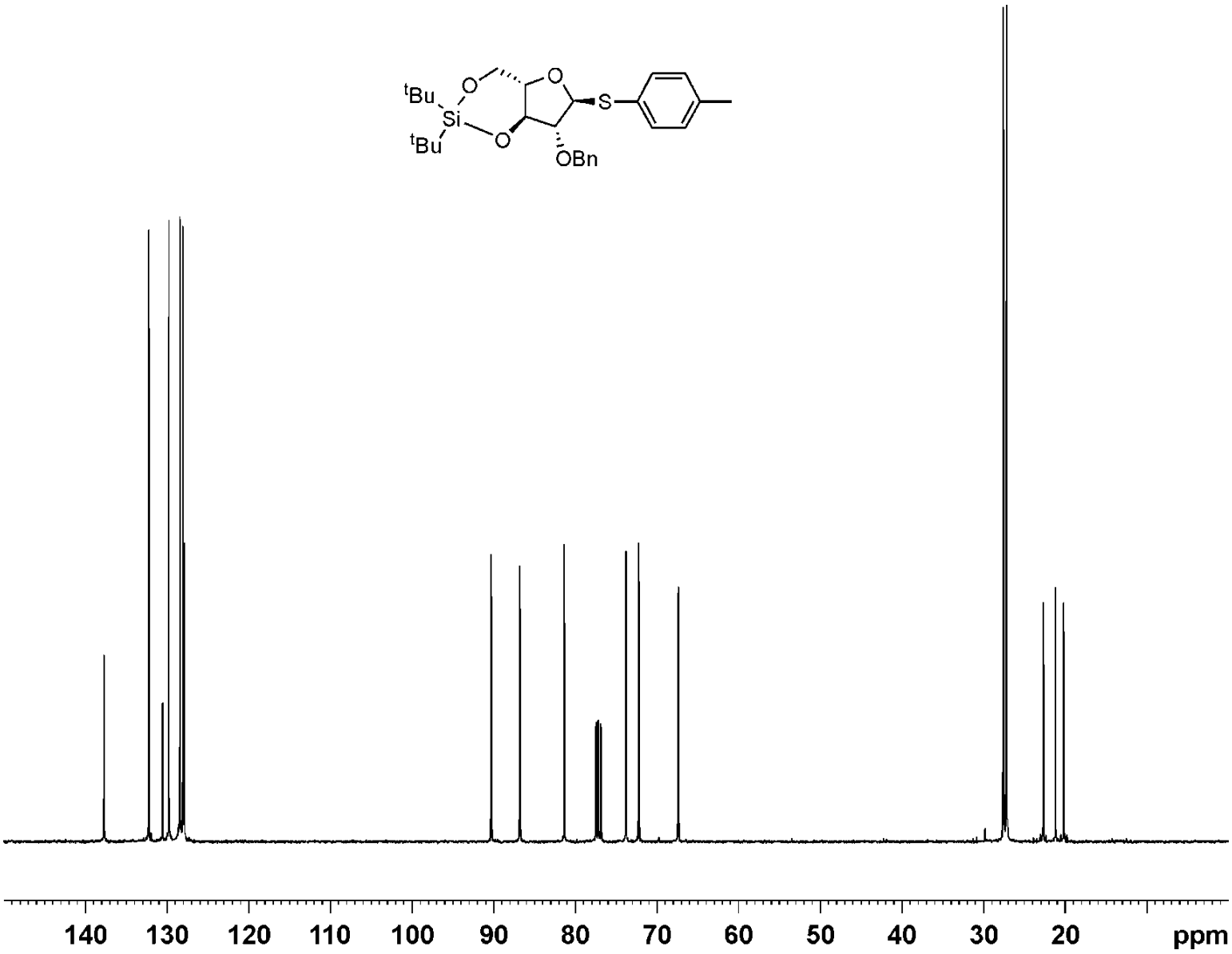


Compound **29** (Scheme 2.12) - ^{1}H NMR spectrum

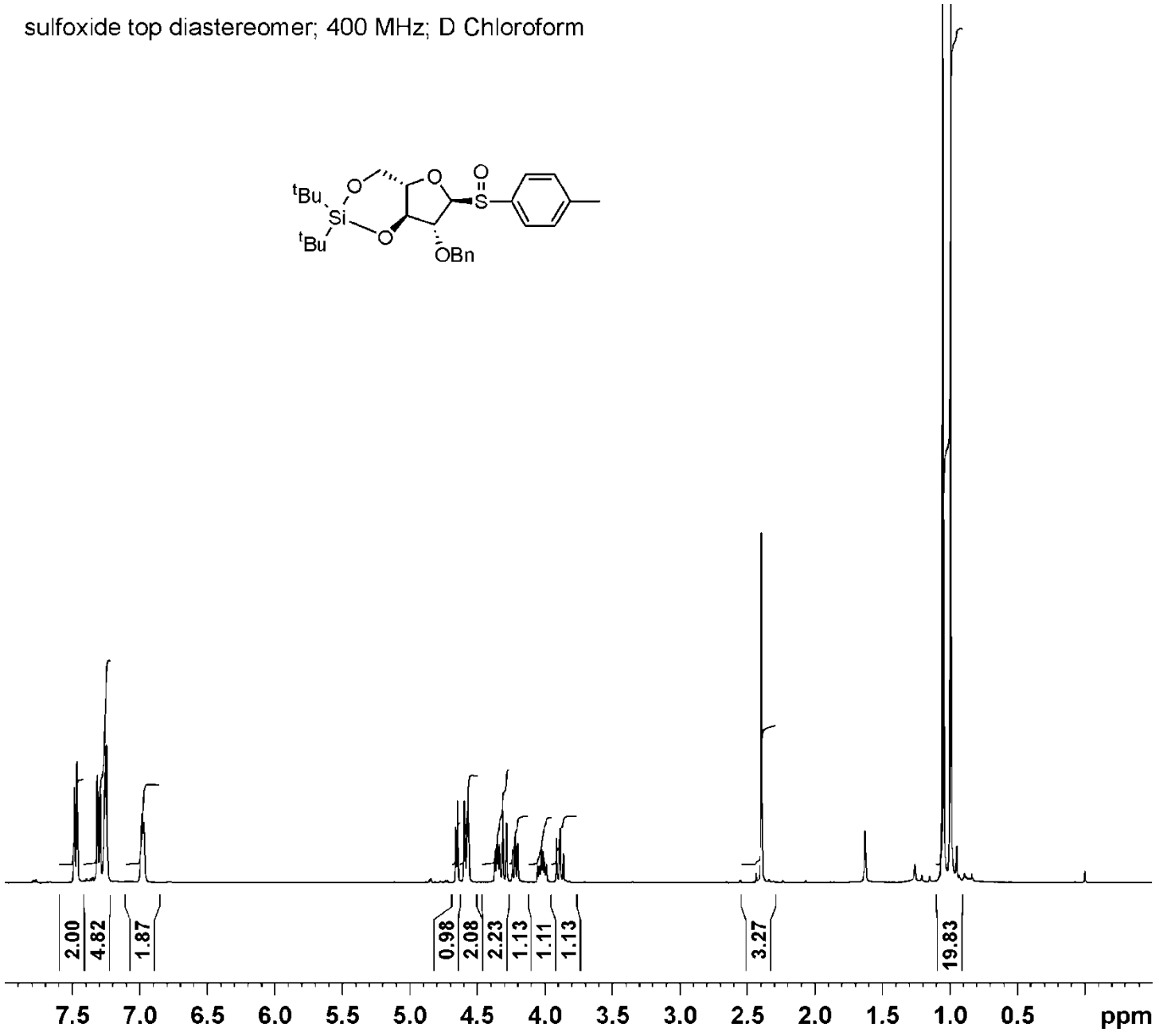


Compound **29** (Scheme 2.12) – ^{13}C NMR spectrum

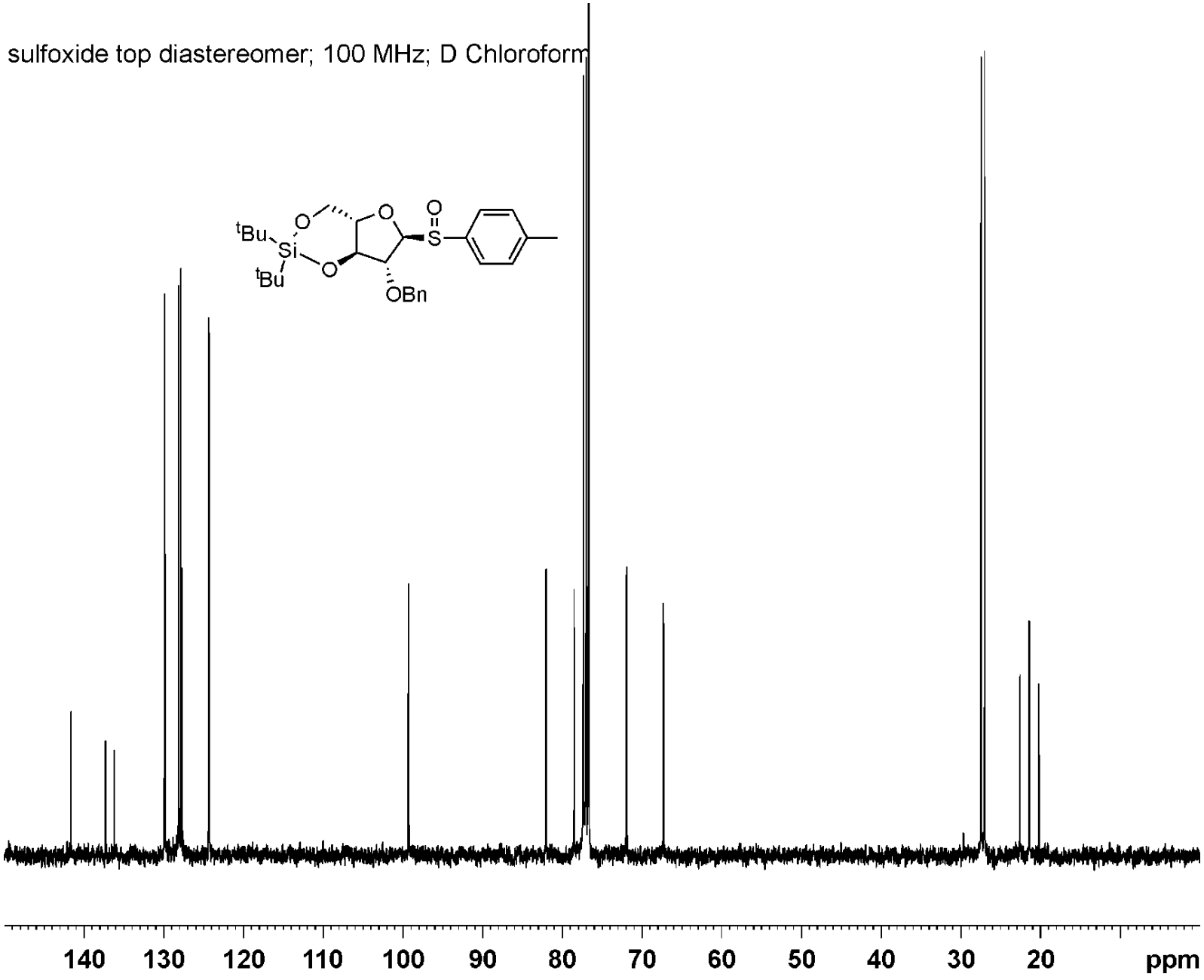
silyl benzyl ether; 100 MHz; D Chloroform



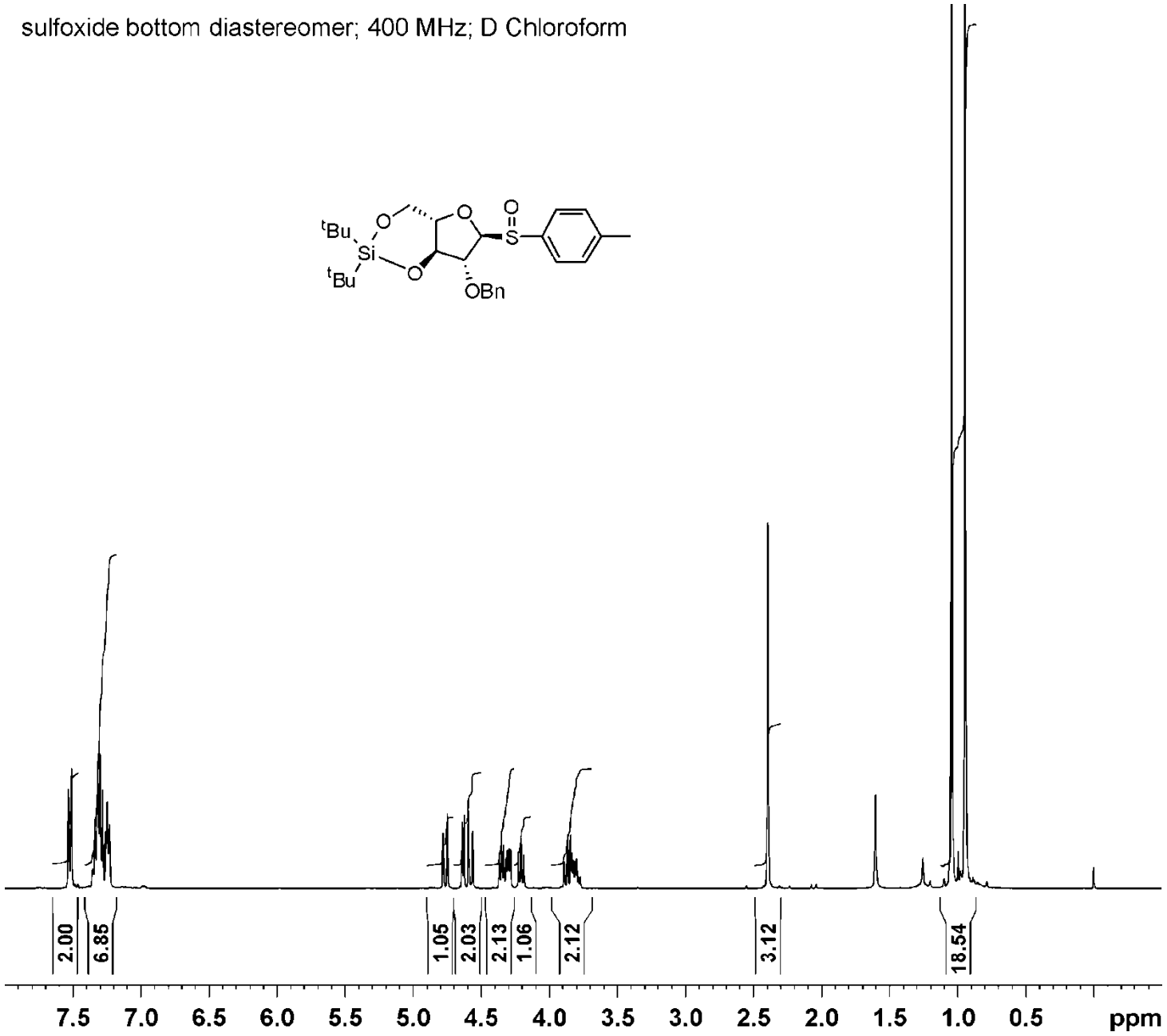
Compound **42a** (Scheme 2.15) - ^{1}H NMR spectrum



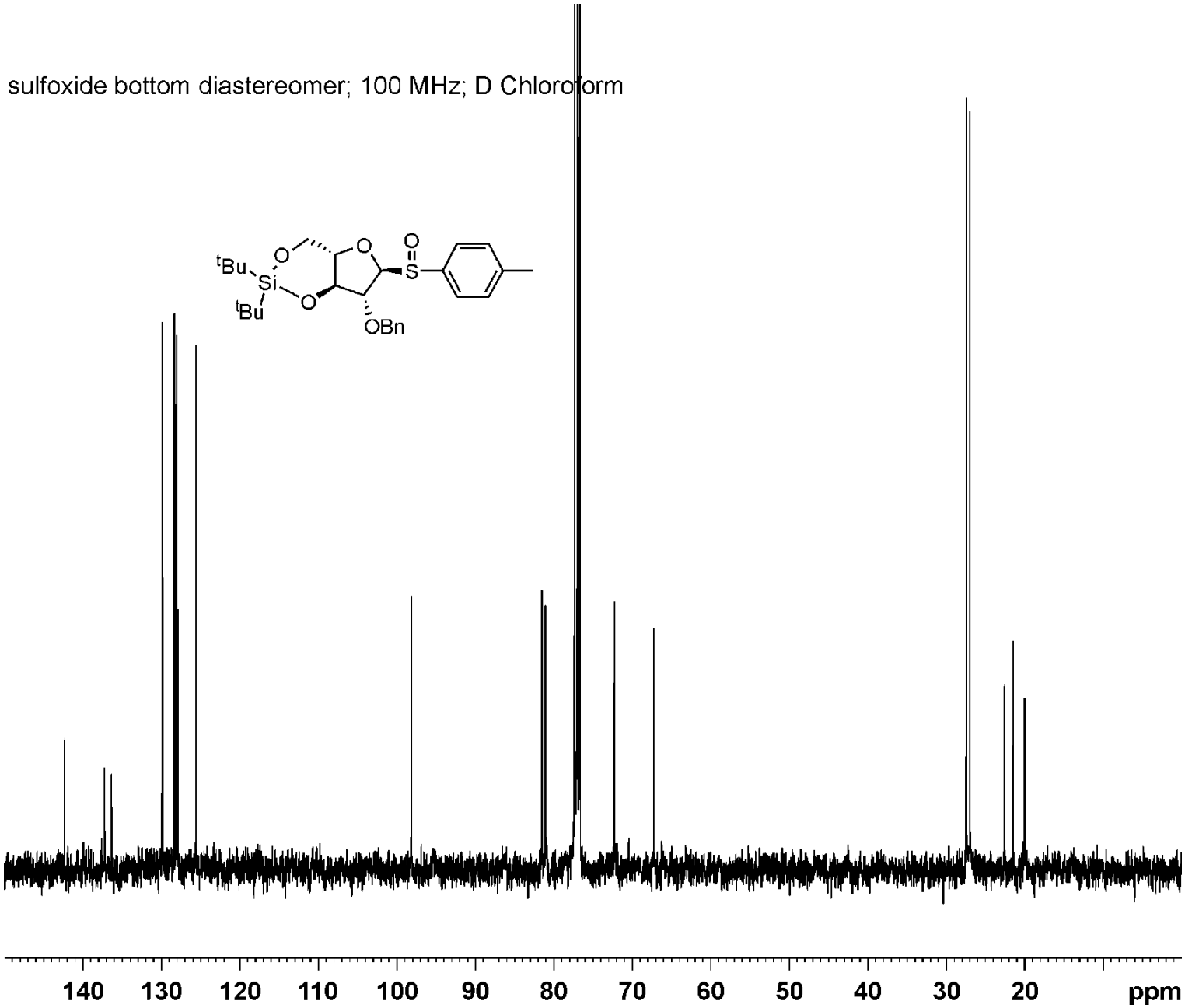
Compound **42a** (Scheme 2.15) – ^{13}C NMR spectrum



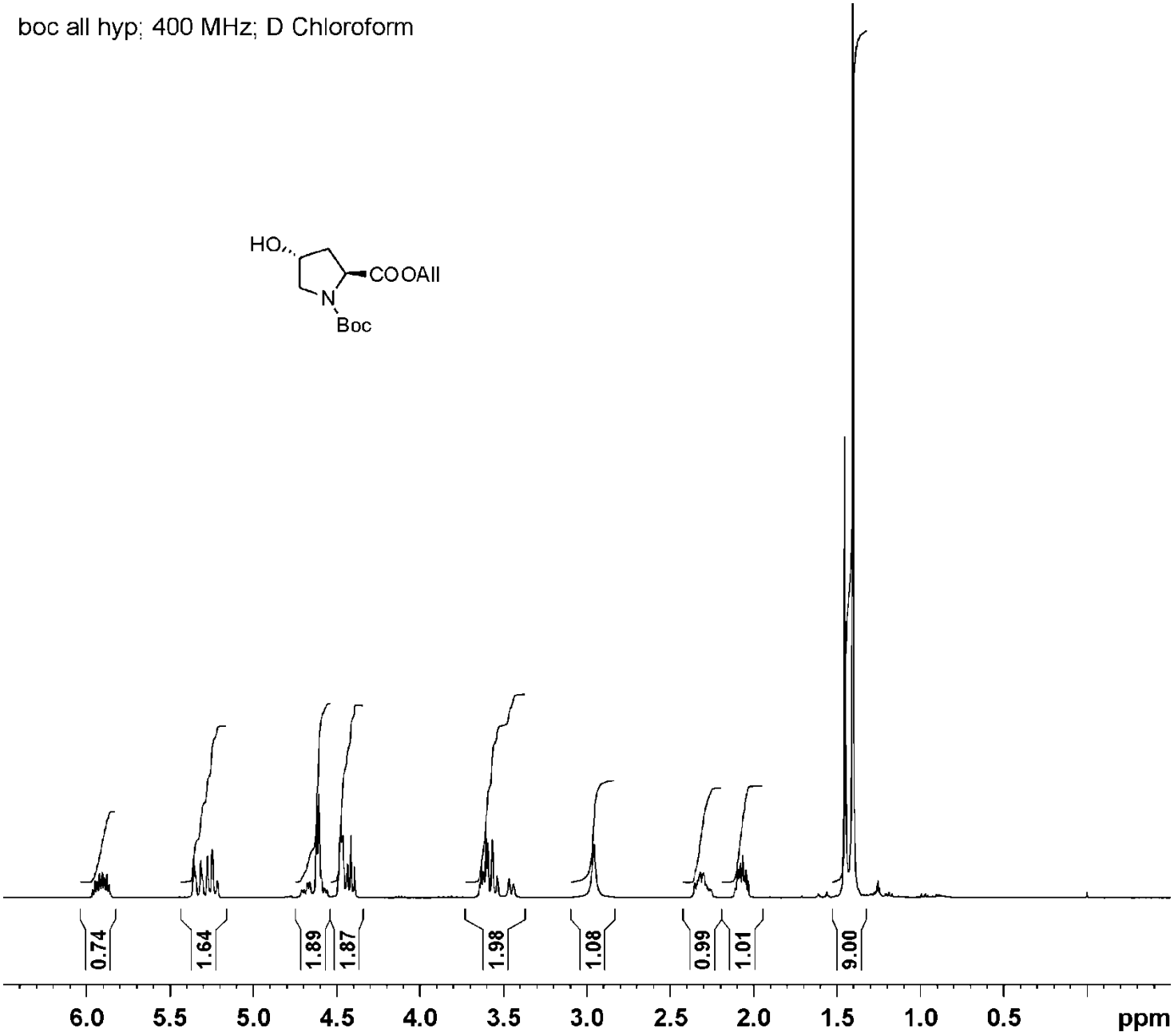
Compound **42b** (Scheme 2.15) - ^{1}H NMR spectrum



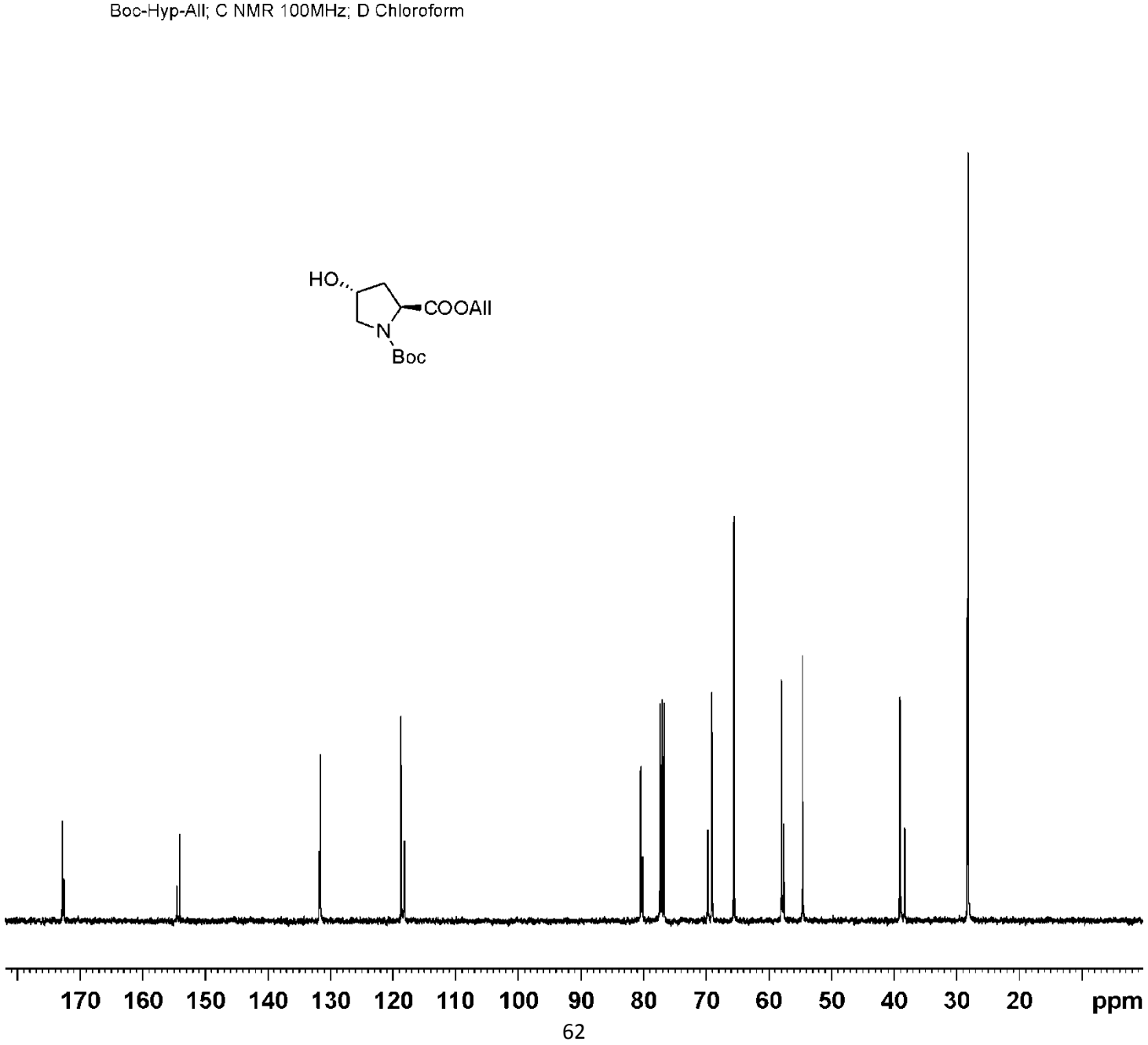
Compound **42b** (Scheme 2.15) – ^{13}C NMR spectrum



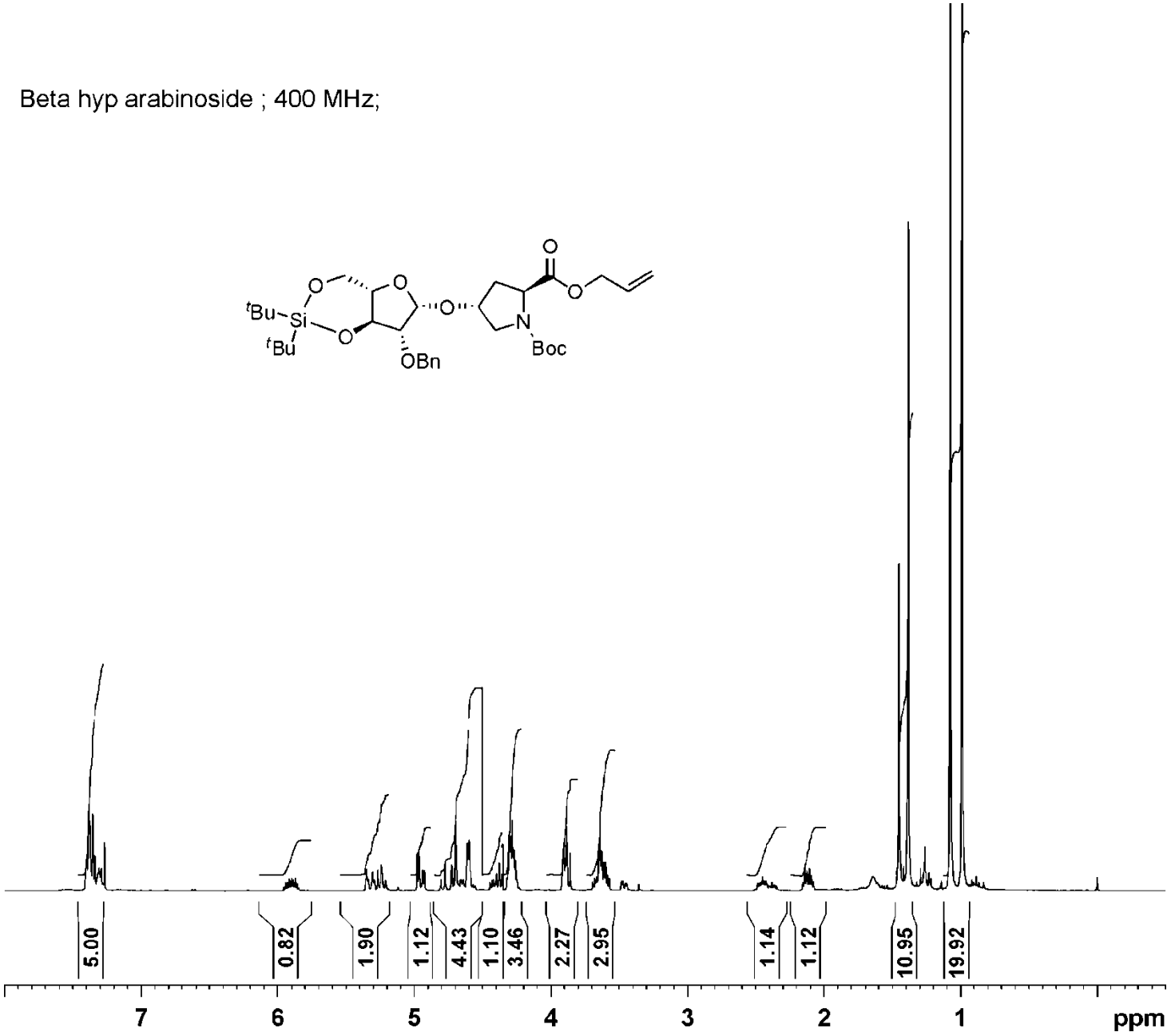
Compound **54** (Scheme 2.13) - ^{1}H NMR spectrum



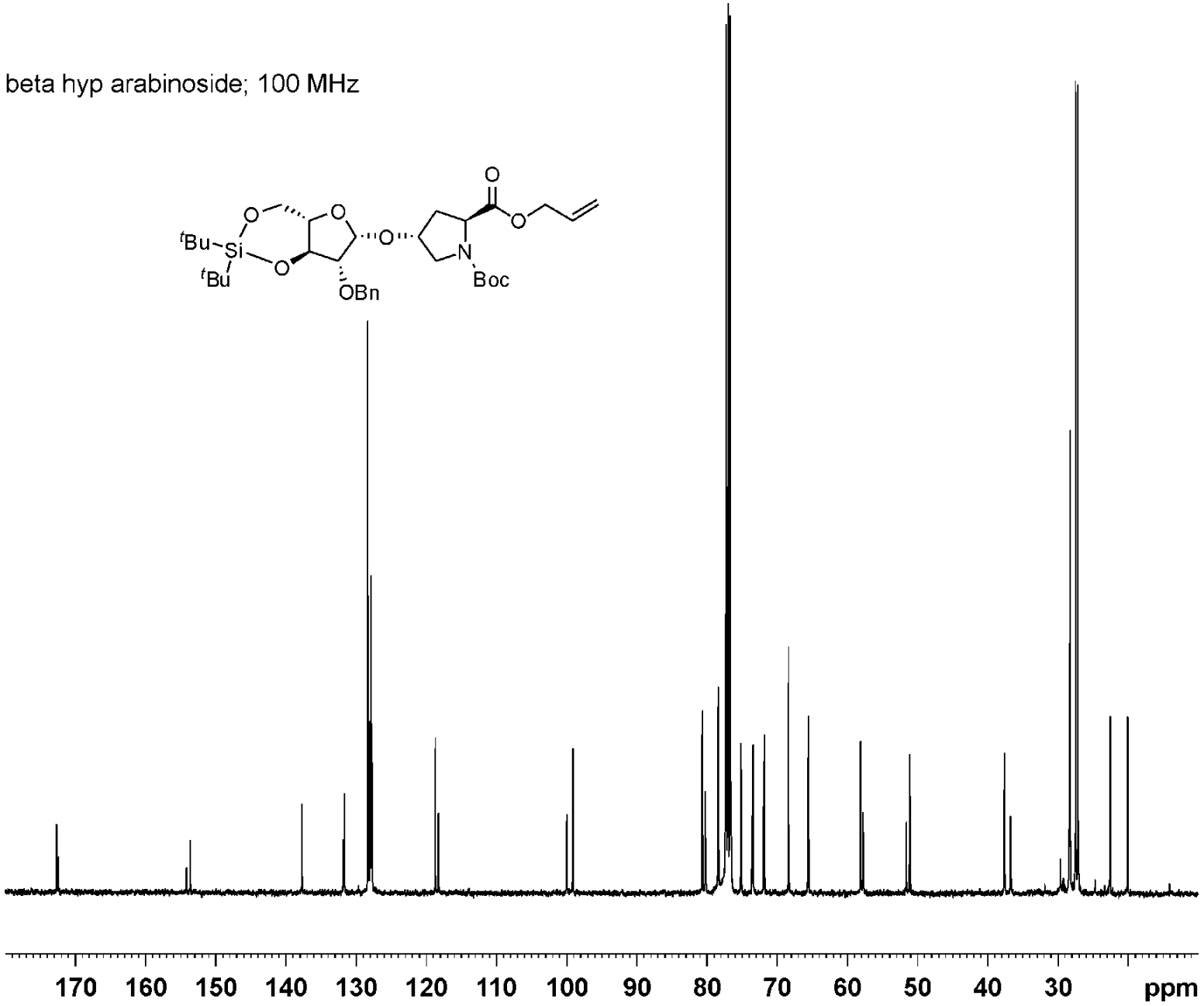
Compound **54** (Scheme 2.13) – ^{13}C NMR spectrum



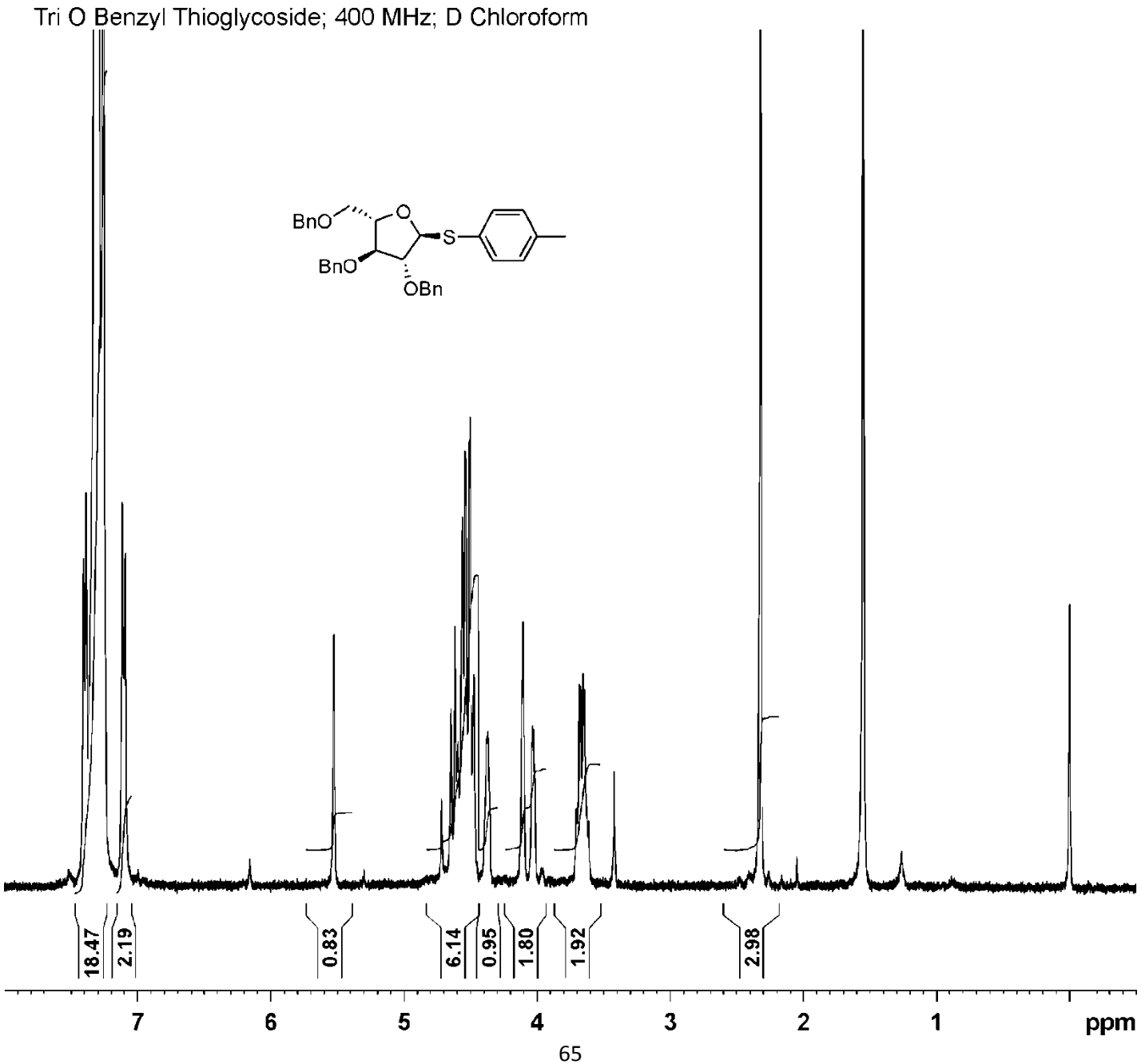
Compound **55β** (Scheme 2.14) - ^{1}H NMR spectrum



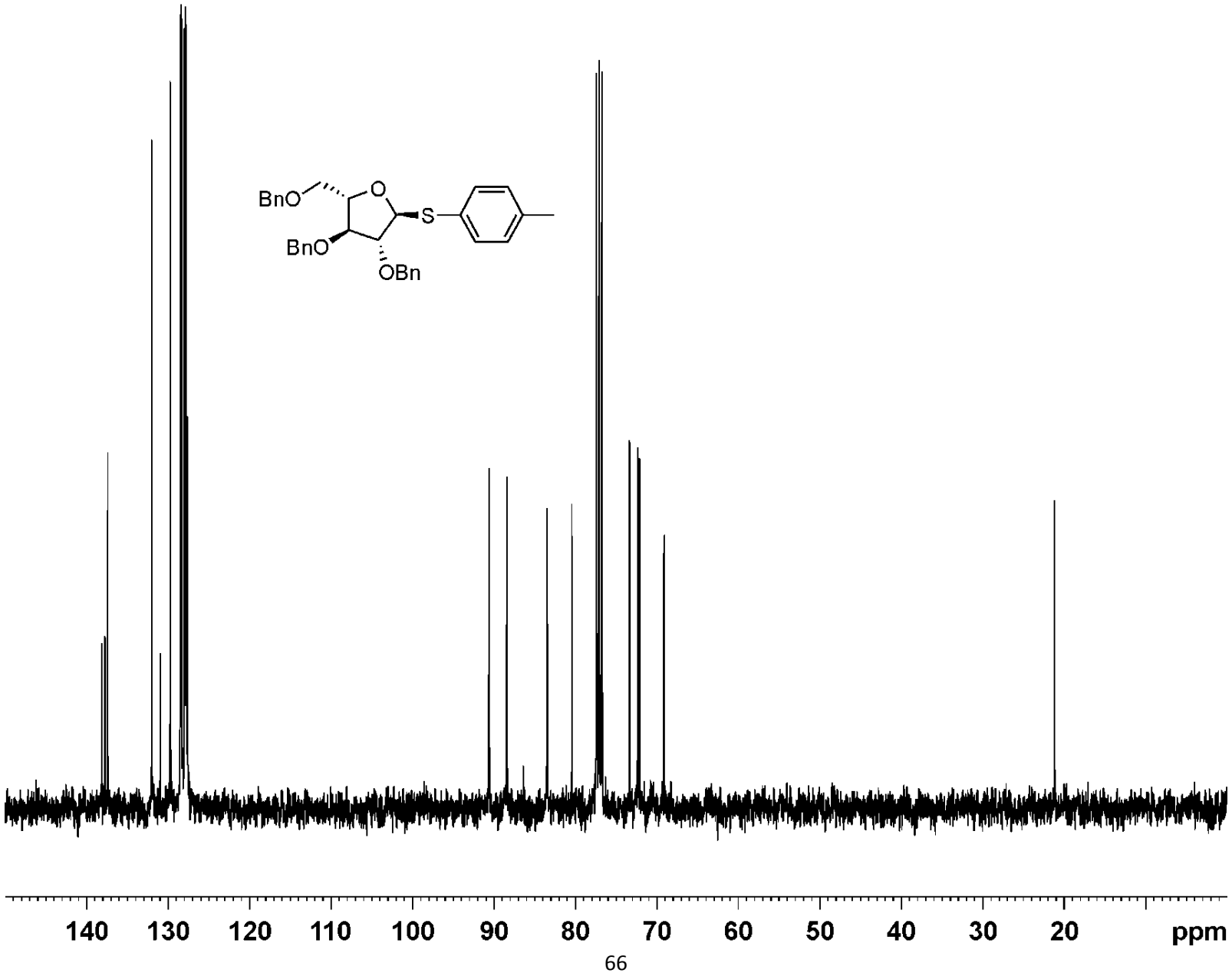
Compound **55β** (Scheme 2.14) – ^{13}C NMR spectrum



Compound **60** (Scheme 2.18) - ^{1}H NMR spectrum

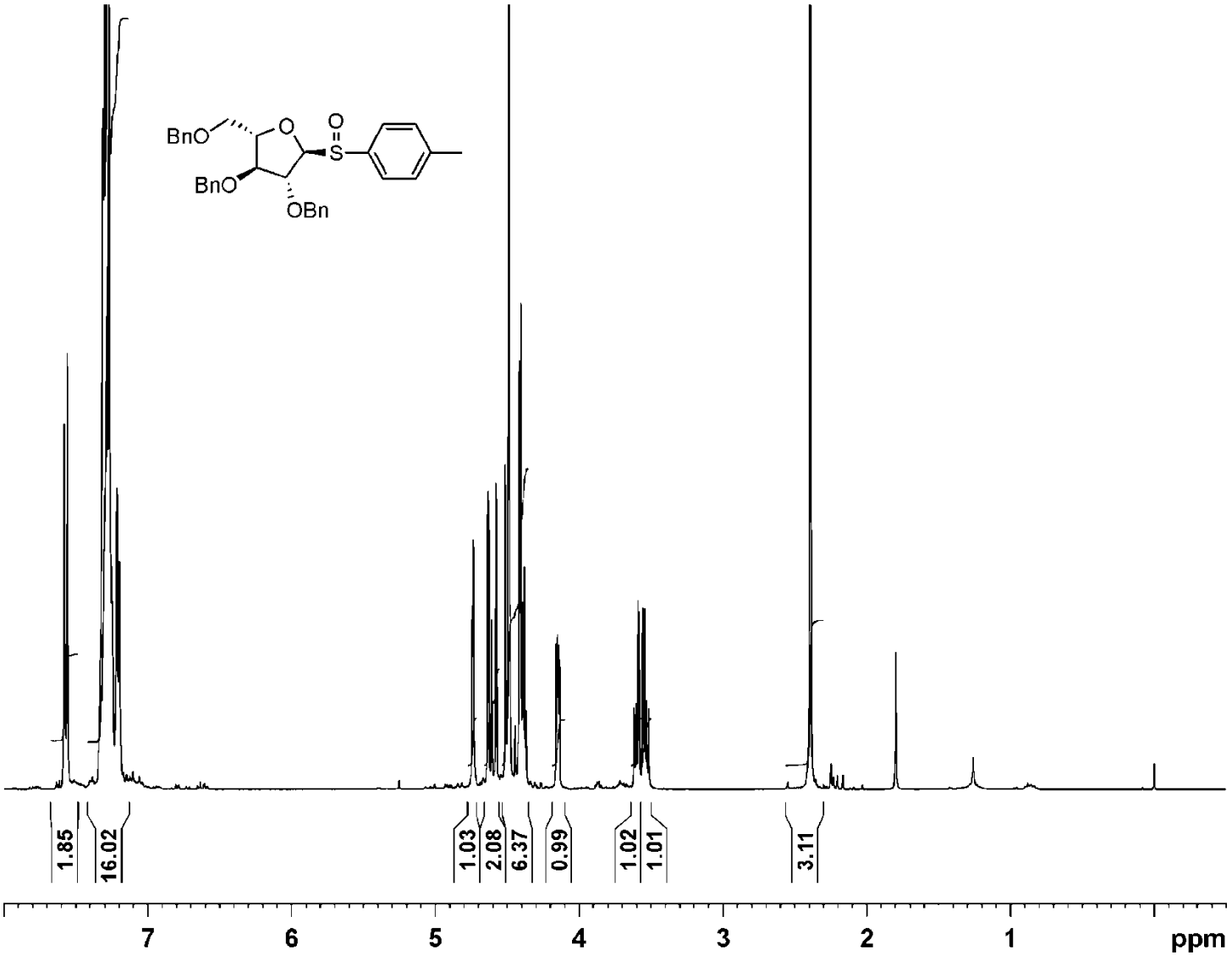


Compound **60** (Scheme 2.18) – ^{13}C NMR spectrum

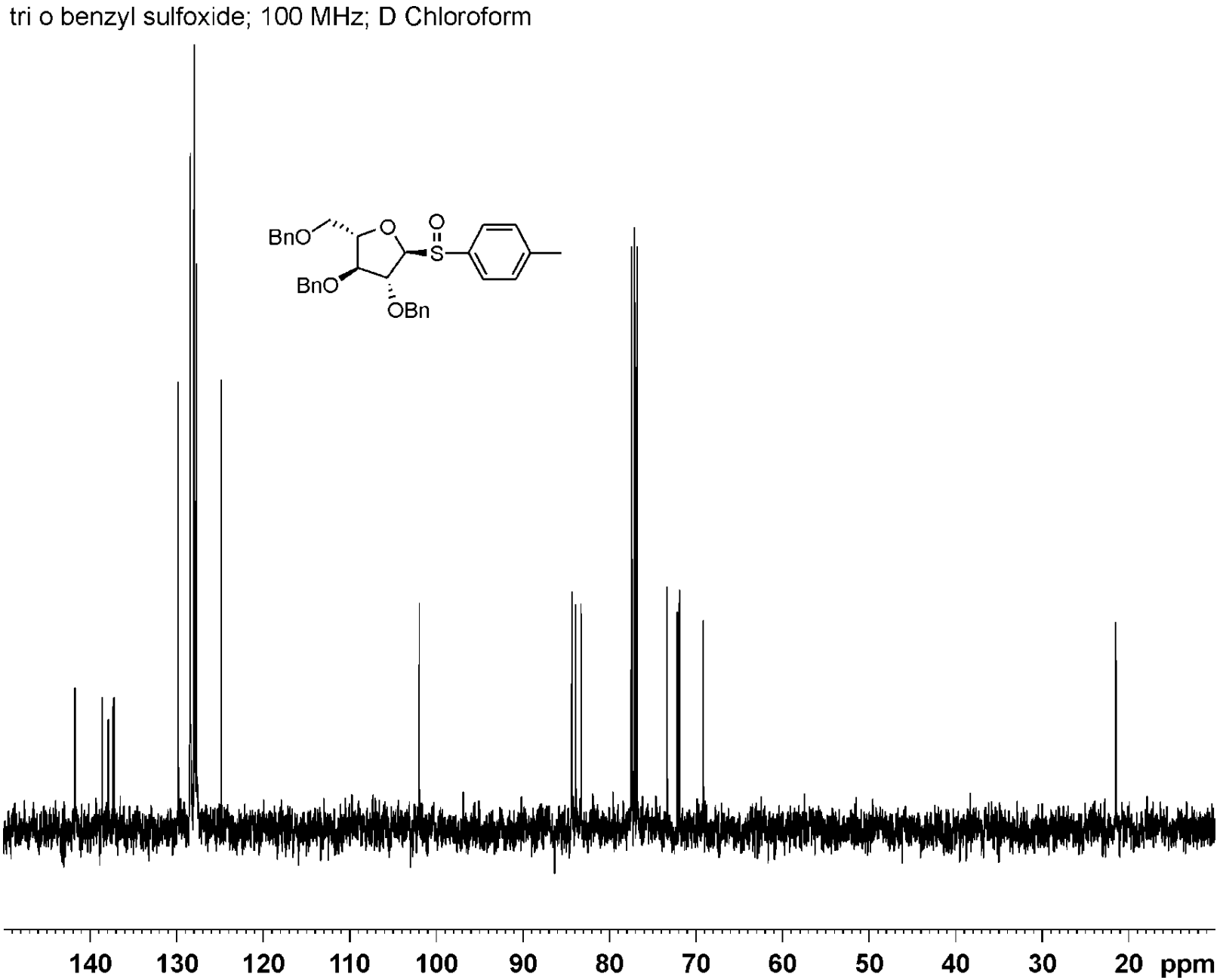


Compound **61** (Scheme 2.18) - ^{1}H NMR spectrum

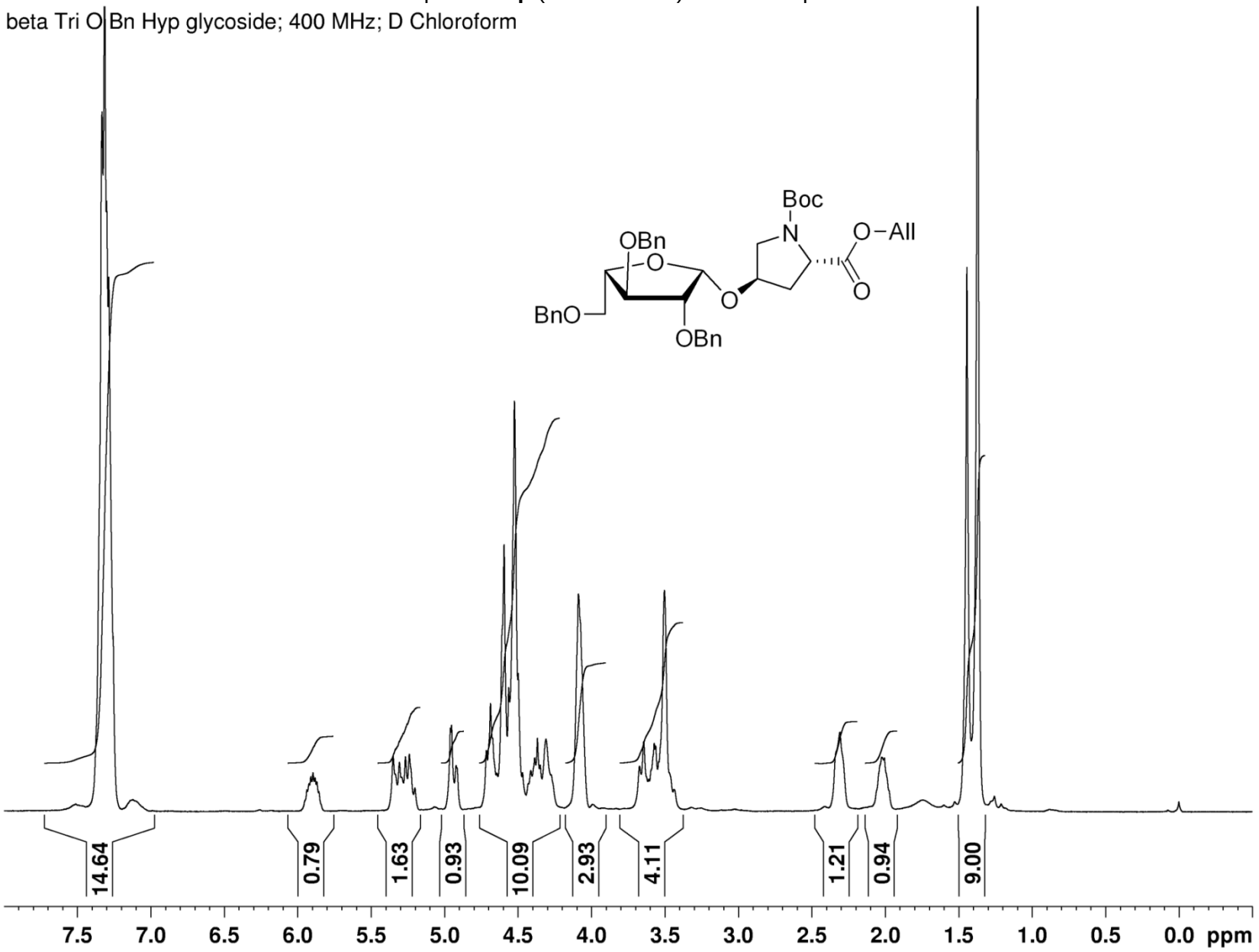
tri o benzyl sulfoxide; 400 MHz; D Chloroform



Compound **61** (Scheme 2.18) – ^{13}C NMR spectrum

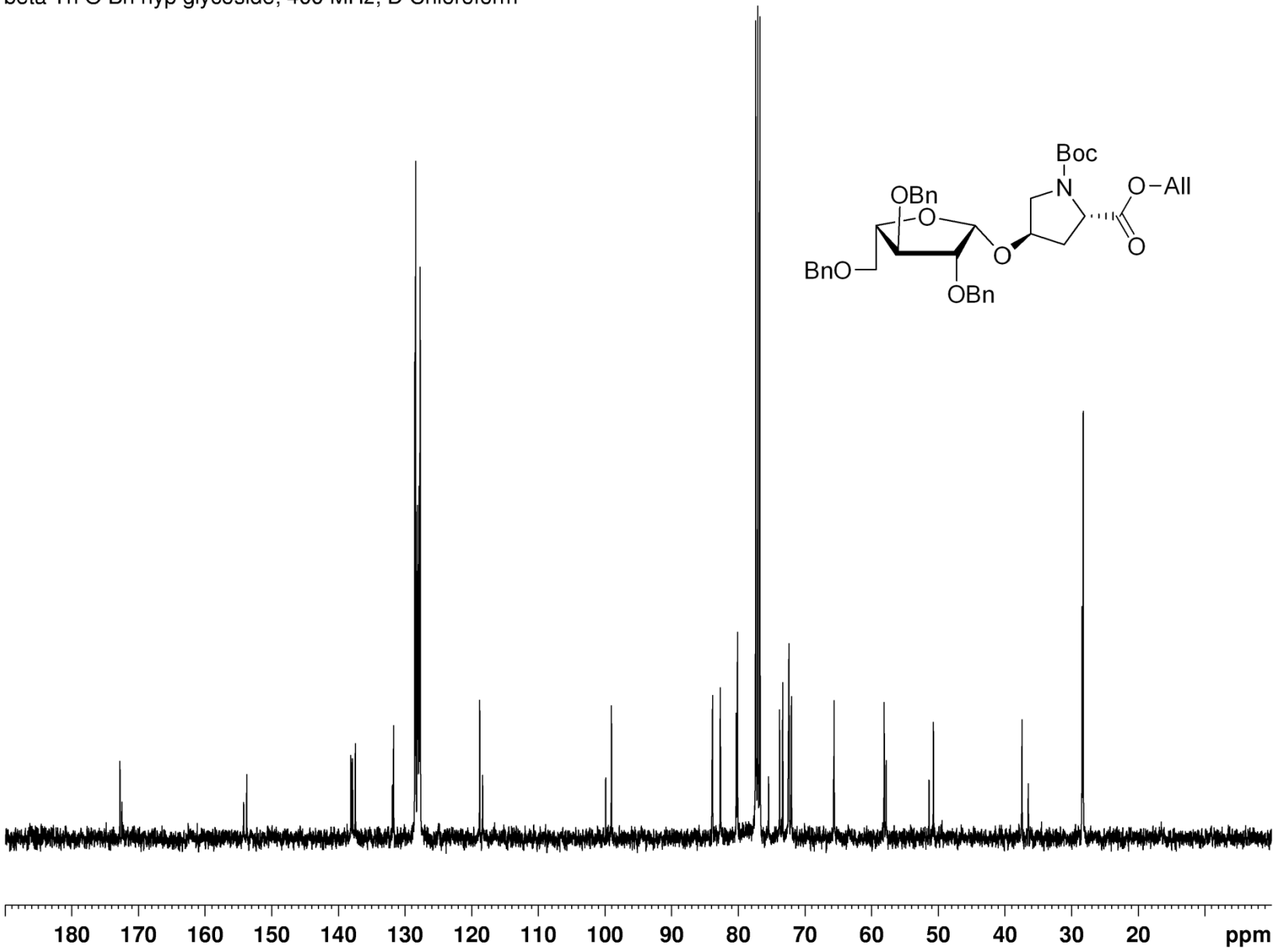


Compound **64β** (Scheme 2.20) - ^{1}H NMR spectrum



Compound **64β** (Scheme 2.20) – ^{13}C NMR spectrum

beta Tri O Bn hyp glycoside; 400 MHz; D Chloroform



CHAPTER 3: SYNTHESIS OF OLIGOMERS OF 4-*O*-[β-*L*-ARABINOFURANOSYL]-(2*S*,4*R*)-4-HYDROXYPROLINE

With the completion of the monomeric β-arabinoside of hydroxyproline in Chapter 2, we moved forward with the assembly of its oligomers. We expected the peptide bond formations to be increasingly difficult due to the gradual accumulation of size in the building blocks and the associated increase in steric demands. We considered a variety of peptide coupling reagents known for their efficiency in reactions involving prolyl amino components. There is an inherent challenge in the coupling of a sterically hindered proline amino component. Fortunately, proline carboxyl components are generally not expected to undergo racemization at C-α, which allowed us to broaden our scope in coupling reagent selection.

3.1 Previous Syntheses of Glycoclusters

There is an abundance of literature detailing synthetic glycocluster peptides.[63] A glycocluster is an array of carbohydrate groups that are present in close proximity as a result of primary sequence or backbone conformation. The "glycoside cluster effect" was introduced by Lee's group to describe the clustering effect that carbohydrate groups exhibit when interacting with protein receptors.[64] While many types of carbohydrate clusters exist, it is the contiguous *O*-glycosylated amino acid cluster that is the focus of the present work. From our perspective, the most relevant solution phase synthesis of these glycoclusters has been in the area of mucins.

The mucin MUC1, a cancer biomarker, is a highly *O*-glycosylated molecule found on the epithelial cells of the stomach, lungs, eyes, and other major organs.[65] In 1998, the Kunz group reported the synthesis of a diglycohexapeptide and a diglycodecapeptide in the investigation of the MUC1 core related glycopeptides (Figure 3.1).[66] These glycopeptides represent two sequences that are prominently found in MUC1 and which are repeated throughout the molecule. Both glycopeptides feature adjacent serine and threonine residues *O*-glycosylated by α-*N*-acetyl galactosamine. The clustered glycopeptide fragment **69** was synthesized by

fragment condensation of Fmoc-[*O*-(2-azido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)]-*L*-Ser-OH (**67**) and H-[*O*-(2-azido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)]-*L*-Thr-Ala-OHep (**68**) (Scheme 3.1).[67] *N*-Hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC) were employed to give the resulting tripeptide in 48% yield.

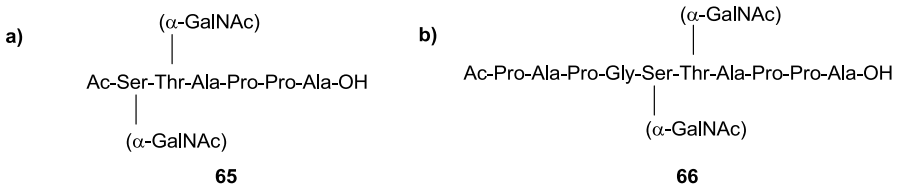
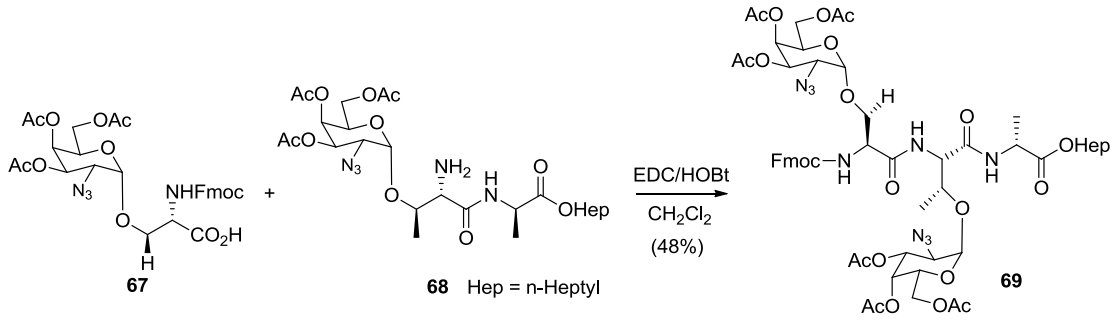


Figure 3.1 MUC1 core related diglycohexapeptide and diglycodecapeptide



Scheme 3.1 Fragment condensation using HOBt and EDC

The Danishefsky group published a paper in 1998 detailing the synthesis of a glycocluster in the T_N and TF (Thomsen-Friedenreich) tumor-associated antigens (Figure 3.2) where the *N*-acetyl galactosamine moiety is α-linked to a serine or threonine residue.[68] The glycosylated amino acids were each unmasked at the *N*- or *C*-terminus and condensed to obtain the diglycodipeptide (**74**/**75**) and, subsequently, the triglycotripeptide cluster (**78**/**79**) (Scheme 3.2). The use of *N*-isobutyloxyl-carbonyl-2-isobutyloxy-1,3,-dihydroquinone (IIDQ) gave great yields in the synthesis of the T_N cluster. However, the bulkier residues, glycosylated by disaccharides in the TF antigen, required the use of HATU/HOAt as the reactions were reported as being "sluggish" with IIDQ.[68] This observation was pertinent to us as the

serine/threonine galactosides have the advantage of being primary amine nucleophiles, thus providing steric advantages in peptide coupling relative to our hydroxyproline arabinosides. In light of this precedent for addressing the steric challenges, we considered that HATU was a strong candidate for the formation of our oligomers.

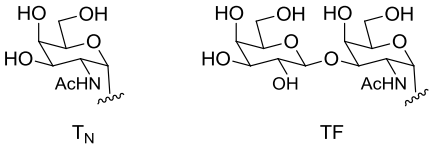
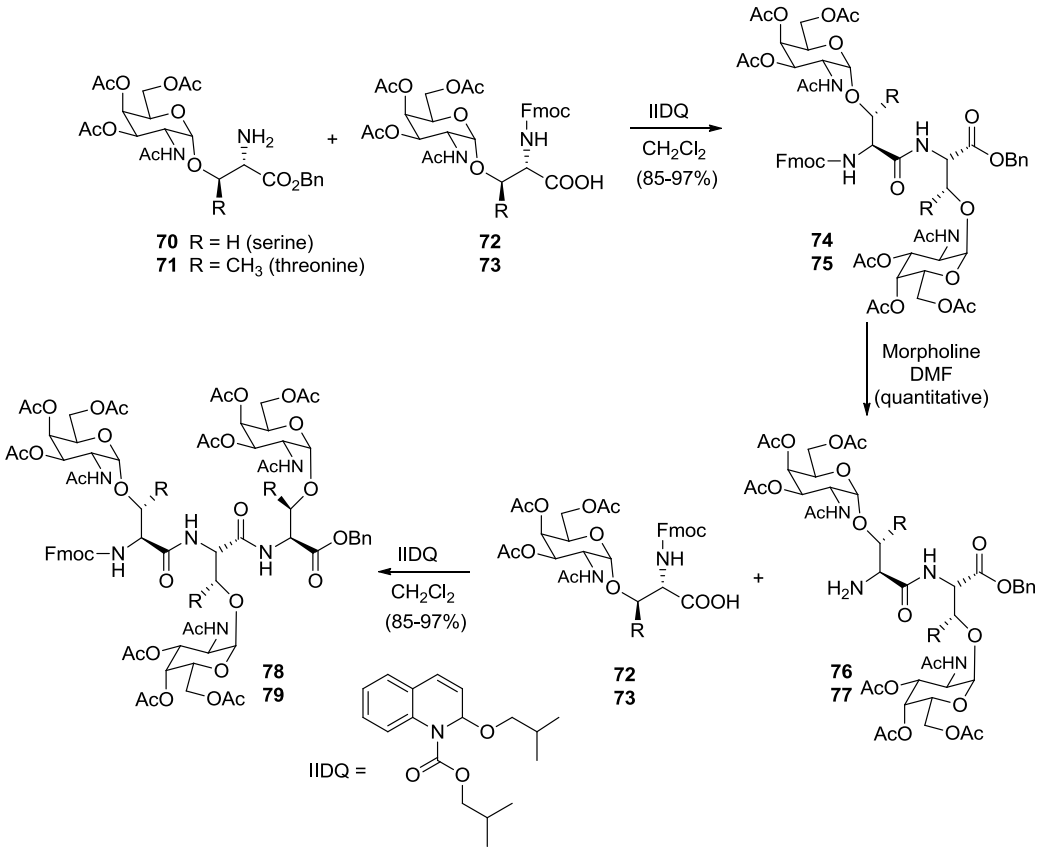


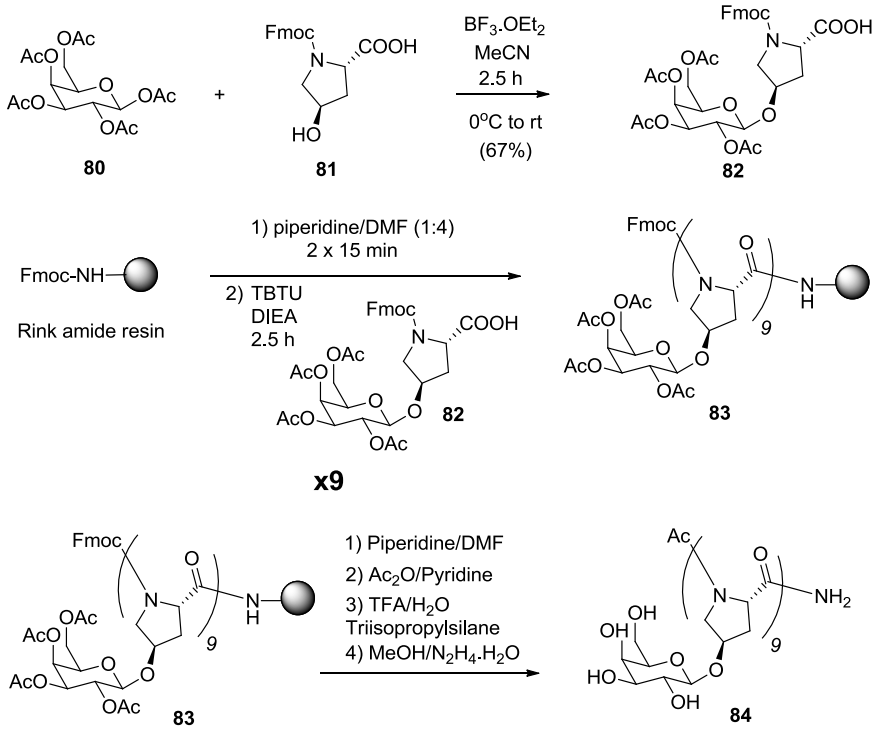
Figure 3.2 T_N and TF antigen



Scheme 3.2 Synthesis of a triglycotripeptide cluster (T_N only)

Contiguous *O*-glycosylated amino acid motifs have also been synthesized using solid phase peptide synthesis (SPPS) by the Barany group to prepare mucin-like glycopeptides[69] and glycopeptide sequences from α-dystroglycan,[70] an extracellular glycoprotein that controls

muscle function. However, the most relevant SPPS study was reported by the Schweizer group.[71] Owens *et al.* investigated the conformational effects of contiguous *O*-galactosylation of *trans*-L-hydroxyproline (details will be more closely examined in Chapter 4). A per-galactosylated nonapeptide (**83**) was prepared by solid phase synthesis using Fmoc-Rink Amide resin; when the peptide is cleaved from the resin it affords a *C*-terminal amide (**84**) (Scheme 3.3). The hydroxyproline galactoside donor was reportedly prepared in one step from commercially available β-D-galactose pentaacetate (**80**) and Fmoc-Hyp-OH (**81**) giving the 1,2-*trans*-glycoside as the only product. For each on-resin peptide coupling, three equivalents of acid **82** was used in combination with four equivalents of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate (TBTU) and eight equivalents of diisopropylethyl amine (DIEA). The authors were able to prepare the nonaglycopeptide (**84**) for their structural studies. However, no yields were reported.

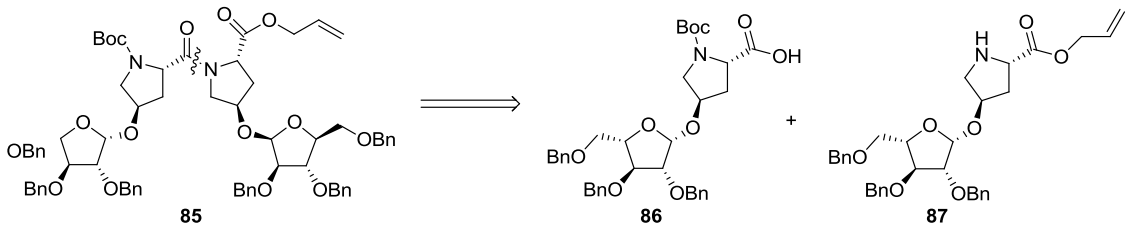


Scheme 3.3 Solid phase peptide synthesis of a nonaglycopeptide

We concluded at the outset that solid phase synthesis was not a realistic route for us due to the high stoichiometry of monomer necessary to carry out each of the cycles. While the galactose pentaacetate donor used by Schweitzer *et al.* is commercially available, our arabinofuranoside donor is not. The difficulty of synthesizing β-L-arabinosides of hydroxyproline, as outlined in Chapter 2, rendered us unwilling to sacrifice the precious material in these excess quantities. Our plan was to utilize the latest advances in solution phase peptide coupling strategies in order to obtain these synthetically challenging glycopeptide clusters in optimal yield and purity.

3.2 Dimer Synthesis

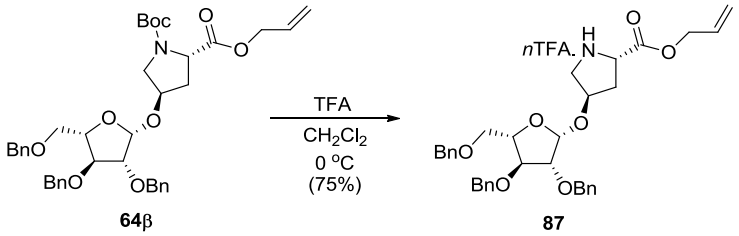
Retrosynthetically, it is obvious that we must derive the free carboxylic acid (**86**) and prolyl amine (**87**) from our hydroxyproline arabinoside monomer for diglycodipeptide (**85)** assembly (Scheme 3.4). Our choice of deprotection methods should look to leave the non-targeted protecting groups unaffected, as well as preserving the ever important *O*-glycosidic linkage.



Scheme 3.4 Retrosynthetic analysis of dimer

Treatment of compound **64β** (Scheme 3.5) with trifluoroacetic acid afforded the secondary amine **87**. When the reaction was carried out at room temperature, we isolated small amounts of a prolyl species lacking the carbohydrate moiety. To minimize cleavage of the *O*-glycosidic linkage, we therefore removed the Boc-carbamate at 0 °C. While the amine salt is typically not purified any further due to its high polarity, we found it beneficial to submit the

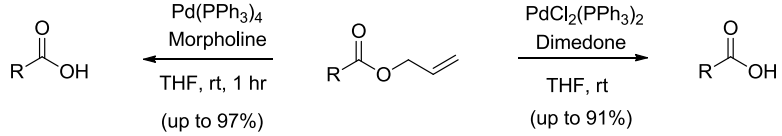
residue to flash chromatography prior to the coupling reaction. Proton and ^{13}C NMR spectra of compound **87** saw the disappearance of the second set of peaks present in the spectra of **64β** as a consequence of rotational isomerization, thus confirming that the two species observed in the spectra of **64β** were indeed assignable to carbamate rotamers.



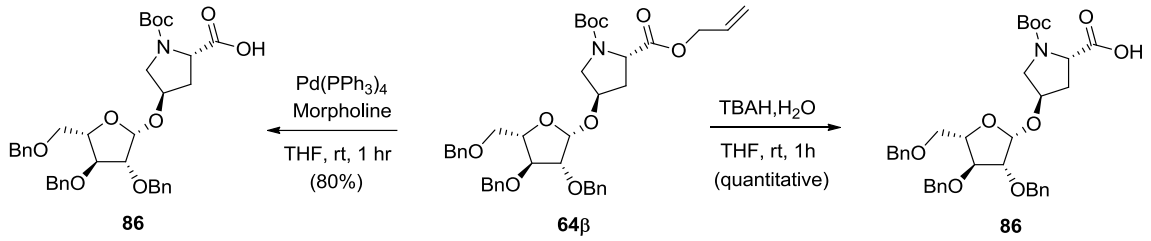
64β **87**

Scheme 3.5 Boc deprotection of monomer

Our initial motivation to mask the acid as an allyl ester was the established ease of deprotection under mild or neutral conditions (Scheme 3.6). Deallylation of the allyl ester with 10 mol % of palladium (0) and an allyl scavenger could be achieved in excellent yield as shown by the groups of Zhang and Kunz.[72] We chose *tetrakis*(triphenylphosphine)palladium(0) and morpholine as the reaction conditions because the scavenger and its allylated counterpart could be purified from the product through simple extraction using dilute HCl. The product, however, could not be rendered free from residual catalyst, as evidenced by the distinct gold color which carried into the dimeric product of coupling. The high polarity of the free acid also made purification by flash chromatography difficult. In search of an ester cleavage protocol free of byproducts, we decided to try mild basic hydrolysis using aqueous tetrabutylammonium hydroxide (TBAH) in THF (Scheme 3.7).[73] The product of this reaction was colorless and was not subjected to flash chromatography despite it being not completely free of byproducts.

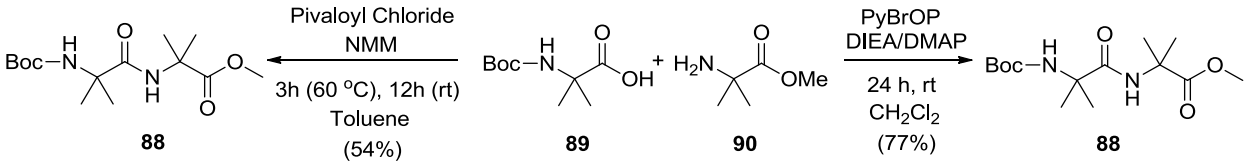


Scheme 3.6 Known methods of allyl ester deprotection



Scheme 3.7 Deallylation of monomer

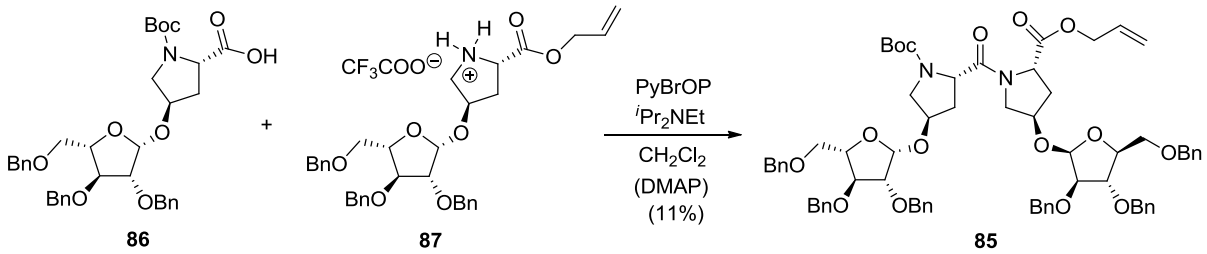
We tried a variety of coupling reagents in the synthesis of the clustered diglycodipeptide. We first looked at bromo-*tris*-pyrrolidinophosphoniumhexafluorophosphate (PyBrOP) for our coupling due to its efficiency in the difficult coupling of the dipeptide of α-aminoisobutyric acid (Aib) (**88**) (Scheme 3.8).[74] Aib is a sterically hindered α-methylalanyl residue that can affect the conformation[75] of a peptide as well as its pharmacological activity. The coupling of Boc-Aib-OH (**89**) and H_2N-Aib-OMe (**90**) was carried out using PyBrOP and 4-dimethylaminopyridine (DMAP) to give a 77% yield of the hindered dipeptide **88**. Previous methods using pivaloyl chloride and NMM, which constructed the dipeptide by way of a mixed anhydride, gave only 54% of product.[76] Considering that both our nucleophile and carboxylate are also fairly hindered, we chose PyBroP as our first coupling reagent.



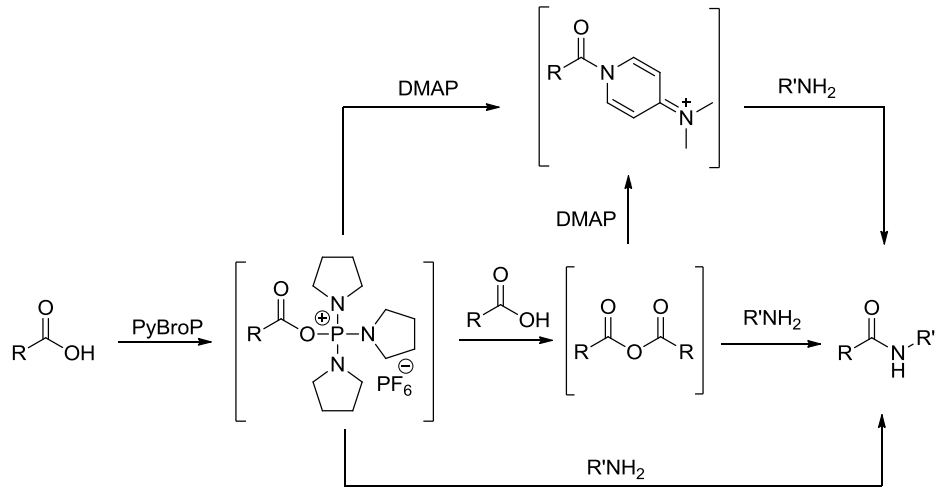
Scheme 3.8 Difficult coupling of a dipeptide of aminoisobutyric acid

Coupling of acid **86** and amine **87** with PyBrOP (Scheme 3.9) yielded unsatisfactory results. Thin layer chromatography (TLC) showed an array of products and the diglycodipeptide (**85**) was isolated in only 11% yield. Unreacted amine and acid starting materials were evident by TLC. Frerot *et al.* showed that addition of DMAP to peptide coupling reactions can improve yields when used in combination with PyBrOP.[74] PyBrOP is known to facilitate the formation of

anhydrides[77] while DMAP promotes the aminolysis of these anhydrides (Scheme 3.10). Unfortunately, the isolated yields of **85** were not much better than coupling without DMAP.



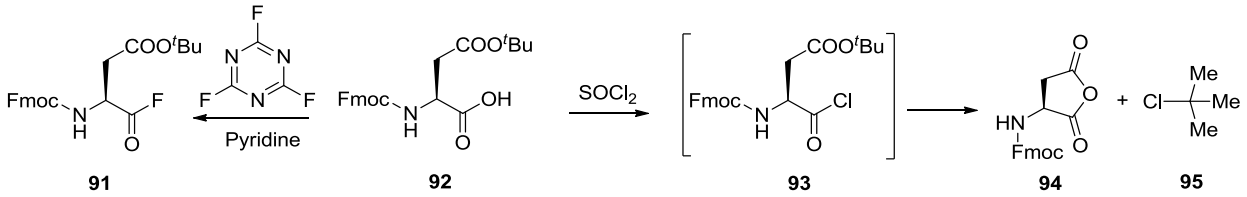
Scheme 3.9 Fragment condensation of dimer with PyBrOP



Scheme 3.10 Mechanism of PyBrOP/DMAP mediated coupling

Given the inefficiency of PyBroP, we moved to a coupling reagent that would generate a less sterically hindered activated species. Carpino and coworkers published a paper in 1990 detailing a new peptide coupling strategy involving the use of stable amino acid fluorides (e.g., **91**) as key intermediates (Scheme 3.11).[78] An amino acid fluoride has an advantage over its chloride counterpart due to the smaller size of the leaving halide. Moreover, amino acid chlorides have shown lability in reactions involving acid side chains containing *tert*-butyl esters wherein formation of the cyclic anhydride **94** and *tert*-butyl chloride (**95**) side products are observed.[79] This instability is not limited to *tert*-butyl esters, however, as β-1-adamantyl ester,

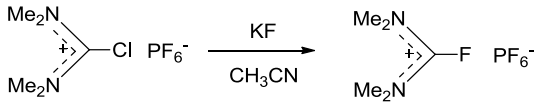
*N*ε-Boc-lysine, and *tert*-butyl ethers of hydroxyl-bearing amino acids also undergo unwanted transformations under coupling conditions involving acid chloride intermediates. Amino acid fluorides do not undergo this process, most likely due to the stronger C-F bond character as compared to its C-Cl counterpart,[80] while being comparable in reactivity toward amine nucleophiles.[81]



91 **92** **93** **94** **95**

Scheme 3.11 Classical preparation of acid fluorides; acid chloride side reactions

Amino acid fluorides were classically synthesized using cyanuric fluoride in pyridine (Scheme 3.11).[82] More recently, tetramethylfluoroformamidinium hexafluorophosphate (TFFH) (Scheme 3.12), a much milder reagent relative to cyanuric fluoride, was used to convert Fmoc amino acids to acid fluorides.[83] Acid fluorides can be generated by treatment of the corresponding acid with TFFH within 15 minutes, with more hindered amino acids taking up to two hours. While these compounds are stable enough to be isolated, peptide coupling reactions can be subsequently carried out in one pot by adding the amine nucleophile and a hindered amine base after formation of the acid fluoride.

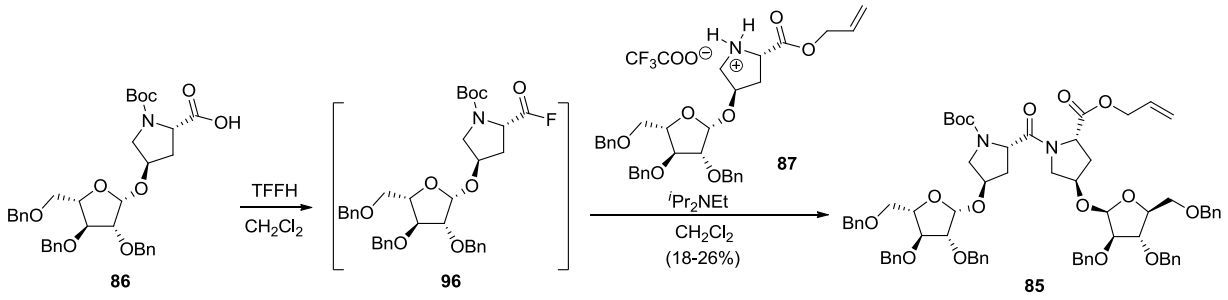


TCFH **TFFH**

Scheme 3.12 Preparation of TFFH

Initial use of TFFH in generating the acyl fluoride of our acid **86** for coupling with the amine monomer **87** gave an increased yield when compared to coupling with PyBroP (Scheme 3.13). Chemical yields ranging from 18-26% were obtained using TFFH. Thin layer chromatography showed that significant amounts of unreacted acid and amine monomers were

present, in common with couplings using PyBrOP. This led us to believe that not all acid monomer was converted to the acyl fluoride **96** or, perhaps once formed, the intermediate underwent hydrolysis by adventitious water. While reaction with TFFH gave yields significantly better than PyBrOP, the results were still unsatisfactory.



Scheme 3.13 Fragment condensation to produce dimer with TFFH

Peptide coupling reagent *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) has been widely used for amide formation in biologically interesting, complex molecules.[84] HATU has proven to be superior to its HBTU counterpart[85] due to the neighboring group effect of the aromatic nitrogen (Figure 3.3b).[86] Carpino has shown that the true form of HATU/HBTU to be the guanidinium salts (*N*-form) as opposed to the uronium salts (*O*-form),[87] which was previously believed to be the case. The *O*-isomers have been shown to be more reactive than the *N*-form.

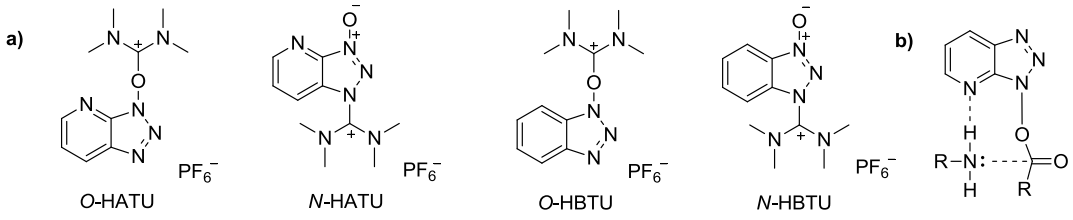
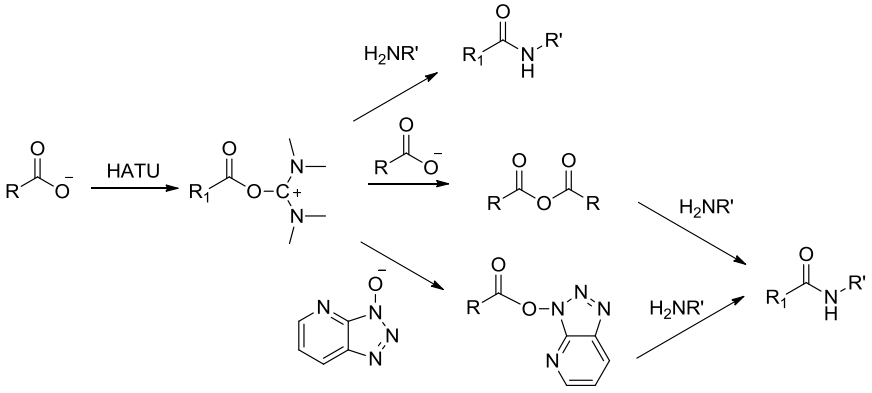


Figure 3.3 a) Guanidinium and uronium salts of HATU and HBTU, b) Neighboring group effect

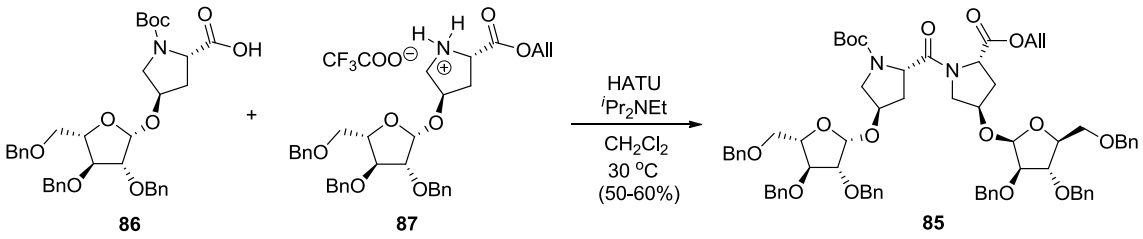
Scheme 3.14 shows the possible pathways of peptide coupling using HATU. Initially, the deprotonated acid nucleophile attacks the electrophilic carbon to form the *O*-acyl urea,

which can immediately couple with the amine nucleophile to give the resulting peptide and urea by-product. At least two other pathways can transpire after initial activation: 1) the *O*-acyl urea can be displaced by another molecule of the carboxylate to form a symmetrical anhydride, 2) formation of the activated ester. The leaving group of either of the intermediates can be displaced by the amine nucleophile to give the desired peptide product.



Scheme 3.14 Pathways of amide formation using HATU

Chemical yields of diglycodipeptide (**85**), obtained using HATU as the coupling reagent (Scheme 3.15), were immediately better than any reagent we have used previously. Yields of 45-50% were achieved when the reaction was run at room temperature. When the reaction was initiated in an ice bath, then allowing the mixture to warm to room temperature overnight as the ice melted, the percent yield was reduced (~25%). This prompted us to investigate the effects that increasing the temperature might have on this coupling reaction. Interestingly enough, mild heating of the reaction vessel (30 °C) gave higher percent yields (50-60%). Further increase in temperature did not show any improvement in chemical yield. Optimization of this reaction condition has allowed us to obtain the dimer in amounts practical for the further synthesis of larger oligomers. The diglycodipeptide has been fully characterized by mass spectrometry, ^{1}H and ^{13}C NMR, and 2-D NMR experiments. Structural studies will be elaborated in Chapter 4.

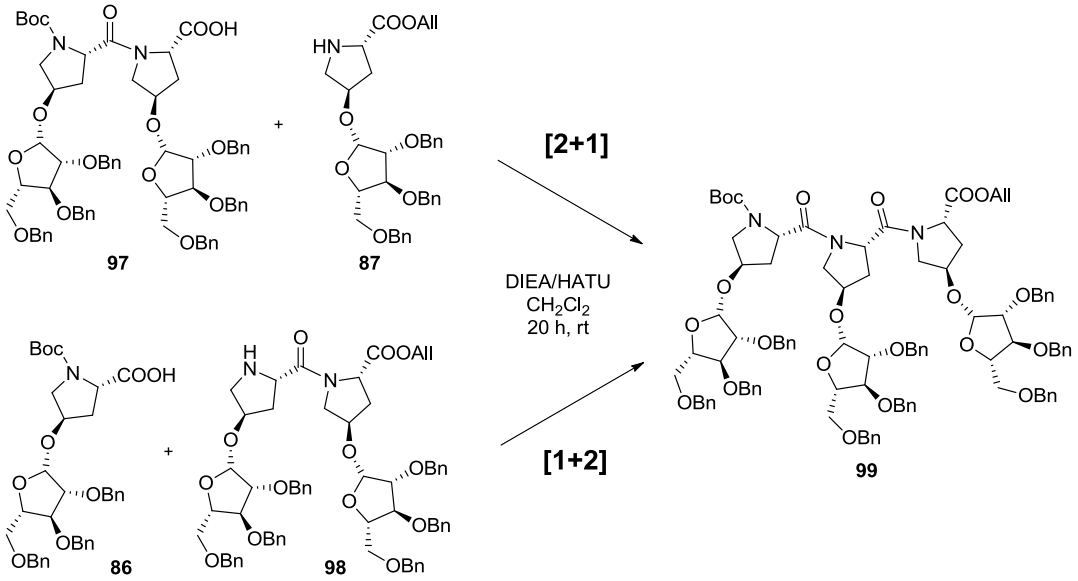


86 87 85

Scheme 3.15 Fragment condensation to produce dimer using HATU

3.3 Oligomer Synthesis

In the preparation of the trimer **99**, we were faced with two possible paths to reach the glycopeptide: a [2+1] or a [1+2] coupling strategy. The [2+1] coupling would place the steric bulk of an extra hydroxyproline arabinoside residue on the free acid **97** in favor of a less sterically hindered amine nucleophile **87**, while the [1+2] strategy would feature a bulkier prolyl nucleophile **98** coupling to a less sterically hindered acid **86** (Scheme 3.16).

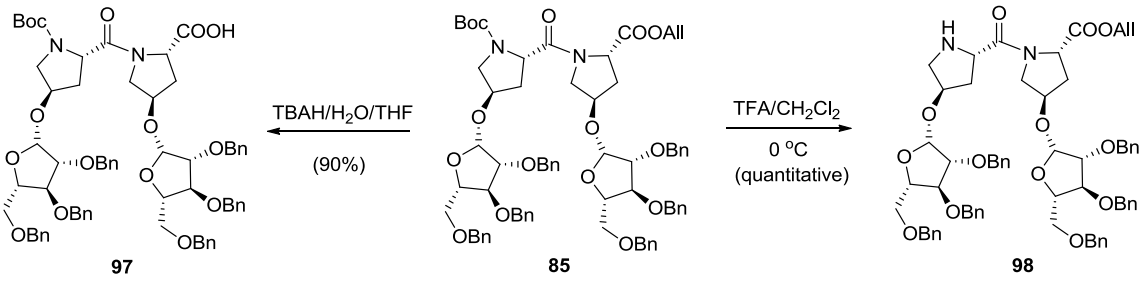


97 87 86 98 99

Scheme 3.16 [2+1] and [1+2] Trimer coupling strategy

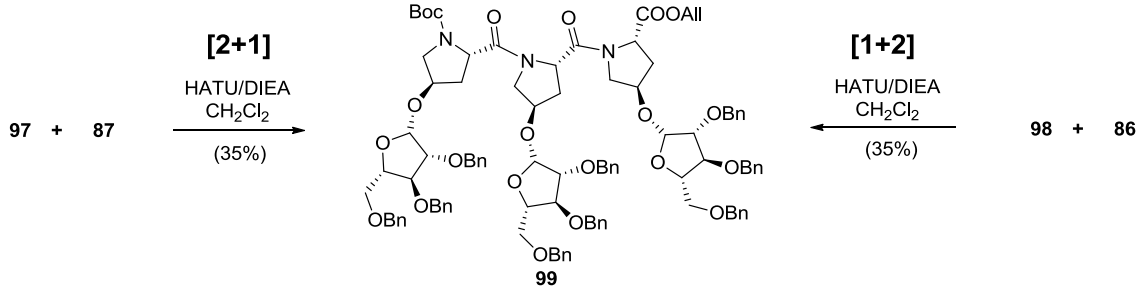
Free acid **97** and amine **98** were prepared from diglycodipeptide **85** (Scheme 3.17). Tetrabutylammonium hydroxide was again used to hydrolyze the allyl ester to avoid the need for

chromatography as the polarity of free dimer acid **97** is higher than that of its monomer counterpart. The heightened polarity likely also accounts for the less than quantitative yields as the dimer acid has a higher affinity for the aqueous layer during work-up. The free amine **98** could still be obtained quantitatively with 50% TFA in dichloromethane at 0 °C.



Scheme 3.17 Preparation of dimer building blocks

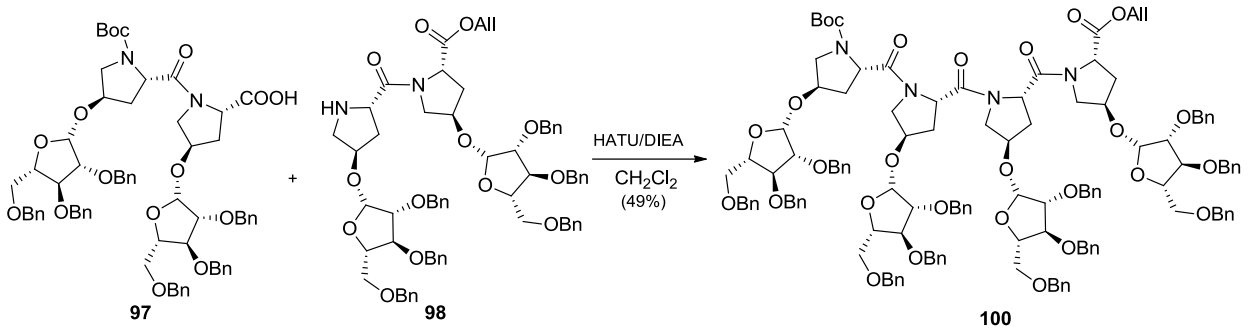
The [2+1] coupling of dimer acid **97** and monomer amine **87** gave the triglycotripeptide **99** in ~35% yield (Scheme 3.18). Alternatively, the [1+2] coupling of monomer acid **86** and dimer amine **98** gave a similar yield. In this instance, raising the temperature slightly did not improve the yield in a noticeable fashion.



Scheme 3.18 Fragment condensation to produce trimer

Confronted with low yields for the construction of the triglycotripeptide, we chose not to use it as a precursor to the tetramer. Instead, the alternate path of a [2+2] coupling, utilizing two dimer building blocks which could be synthesized in reasonable quantities, was pursued. Thus, free acid dimer **97** was coupled to dipeptide amine **98** to afford the glycotetrapeptide in 49%

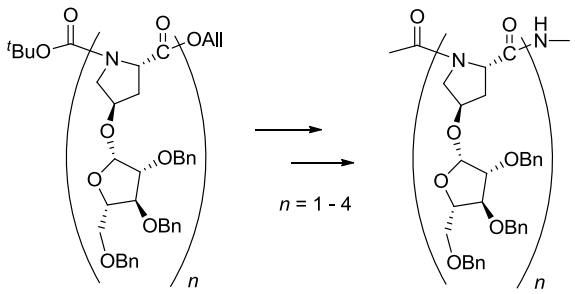
yield (Scheme 3.19). Mass spectrometry, proton, and carbon NMR confirmed the formation of the tetramer (**100**).



Scheme 3.19 Fragment condensation to produce tetramer

3.4 Oligomer End-Capping

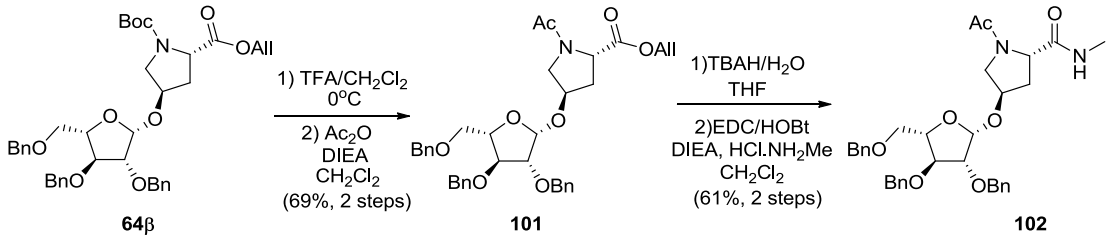
With monomer, dimer, trimer, and tetramer in hand, we began preparations to "end-cap" the glycopeptides. At this stage, the *N*- and *C*-termini were masked as a Boc carbamate and an allyl ester respectively. In order to better mimic the extended peptide, we sought to cap both termini as amides before committing to the global deprotection of the benzyl ethers of the carbohydrate moieties (Scheme 3.20).



Scheme 3.20 End-capping of glycopeptides

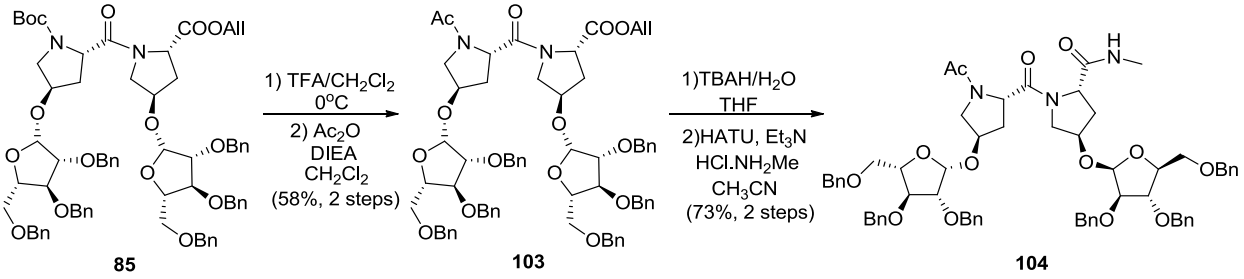
Starting with monomer **64β**, the carbamate was deprotected, as before, using TFA in dichloromethane (Scheme 3.21). Installation of the acetamide with acetic anhydride and diisopropylethyl amine gave compound **101** in 69% yield over two steps. Subsequent deprotection of the allyl ester was carried out under the hydrolysis conditions described

previously. The *C*-terminal methyl amide was formed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/HOBt and methylamine hydrochloride under basic conditions to afford the fully end-capped monomer **102** in satisfactory yield.



Scheme 3.21 Monomer end-capping

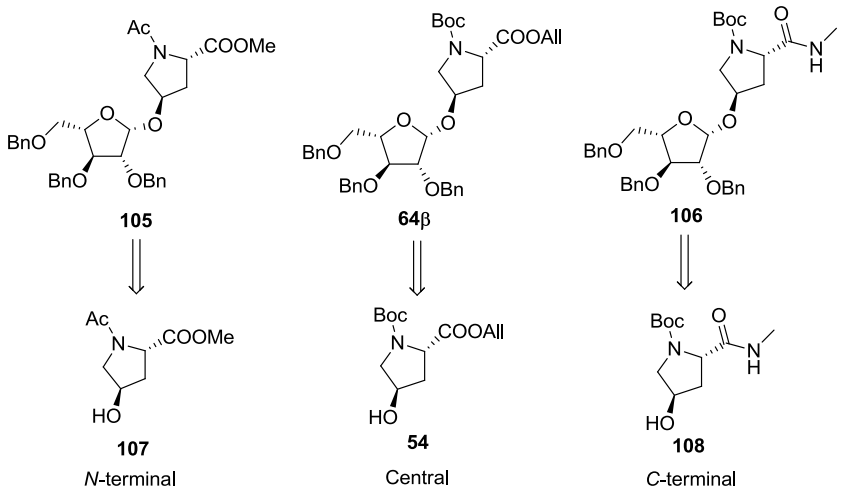
Dimer **85** was treated in a similar fashion to form acetamide **103**, albeit in slightly lower yield, over two steps (Scheme 3.22). Upon deallylation of the free acid, however, we saw a significant drop in yield when using EDC/HOBt as the coupling reagent in the formation of the *N*-methyl amide (**104**). Furthermore, NMR showed that the products obtained were of much lower purity. This problem was rectified by using HATU as the coupling reagent and acetonitrile as the solvent. Quality fully end-capped dimer **104** could be obtained in 73% yield over two steps.



Scheme 3.22 Dimer end-capping

Installation of the *N*- and *C*-terminal amides on the trimer and tetramer proved not to be trivial. Poor yields (trimer: 18% over 4 steps; tetramer: not obtained) forced us to modify our strategy to include end-caps prior to peptide coupling and/or glycosylation. This strategy has the potential to be more convergent, decreasing four linear steps in the overall oligomer

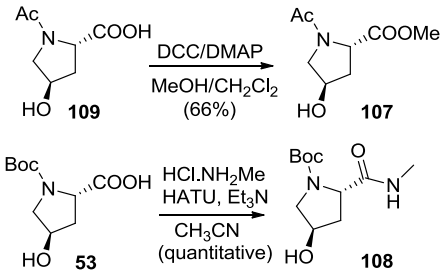
synthesis. This approach called for the preparation of oligomer-specific glycosidic building blocks (Scheme 3.23, **105** and **106**), as well as two new glycosyl acceptors (**107** and **108**).



Scheme 3.23 Oligomer-specific glycosidic building blocks

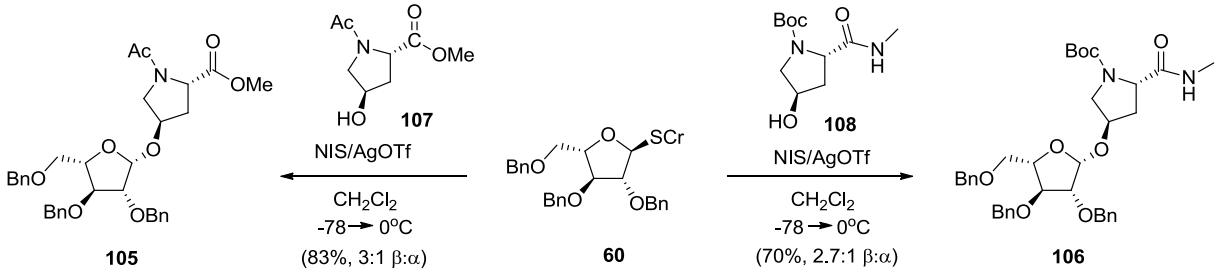
3.5 Pre-End-Capped Glycosides

Glycosyl acceptors **107** and **108** can both be constructed in one step from commercially available *N*-acetyl *trans*-4-hydroxyproline (**109**) and *N*-Boc-*trans*-4-hydroxyproline (**53**) respectively (Scheme 3.24). Treatment of Ac-Hyp-OH with dicyclohexylcarbodiimide (DCC) and 4-DMAP in methanol/dichloromethane affords acceptor **107** in 66% yield. The *N*-methyl amide acceptor **108** could be obtained in quantitative yield using freshly recrystallized methylamine hydrochloride, HATU, and triethylamine in acetonitrile (Scheme 3.24).



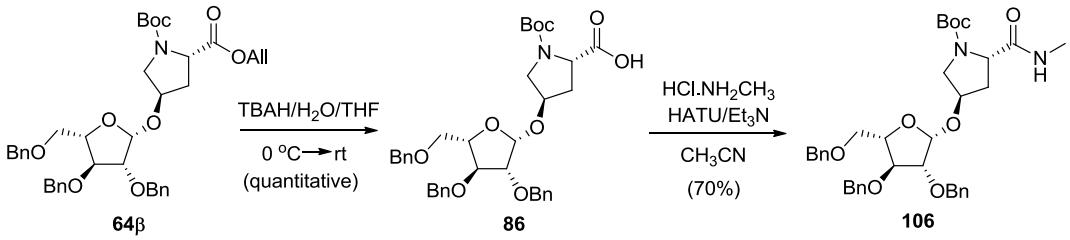
Scheme 3.24 Pre-end-capped glycosyl acceptors

Glycosylation of Ac-Hyp-OMe (**107**) with thioarabinoside donor **60** proved to be high-yielding relative to Boc-Hyp-OAll (Scheme 3.25). While the β:α ratio suffered slightly (3:1 as opposed to 4:1), the overall yield of the target β-glycoside was significantly higher (83% vs 60%). The compromise in selectivity *in lieu* of percent yield did lead to a higher overall yield for the β-glycoside.



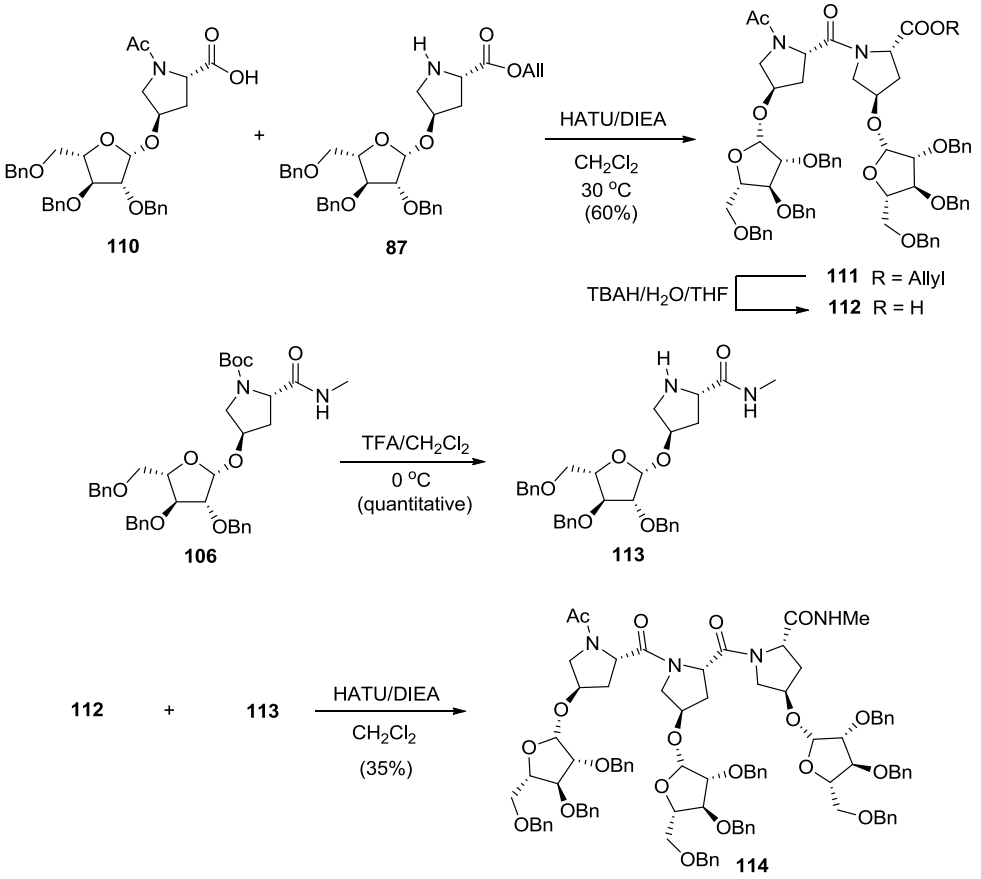
Scheme 3.25 Preparation of pre-end-capped monomers

Glycosylation of Boc-Hyp-NHMe (**108**) with donor **60** under standard conditions gave the methylamide building block **106** in 70 % yield, 2.7:1 β:α ratio. A huge drawback of this reaction is the insufficient polarity differences between the α- and β-anomers. The R_f values of the two diastereomers were nearly indistinguishable, which made separation by flash chromatography all but impossible. We require these building blocks to be synthesized on gram-scale, thus the tedious separation of the anomers by HPLC was impractical. Hence, for this particular building block, we chose to install the methyl amide post-glycosylation on the β-monomer **86** (Scheme 3.26). This transformation can be carried out in two steps from **64β** to give **106** in good yield.



Scheme 3.26 Alternate strategy for preparation of *C*-terminal amide glycoside

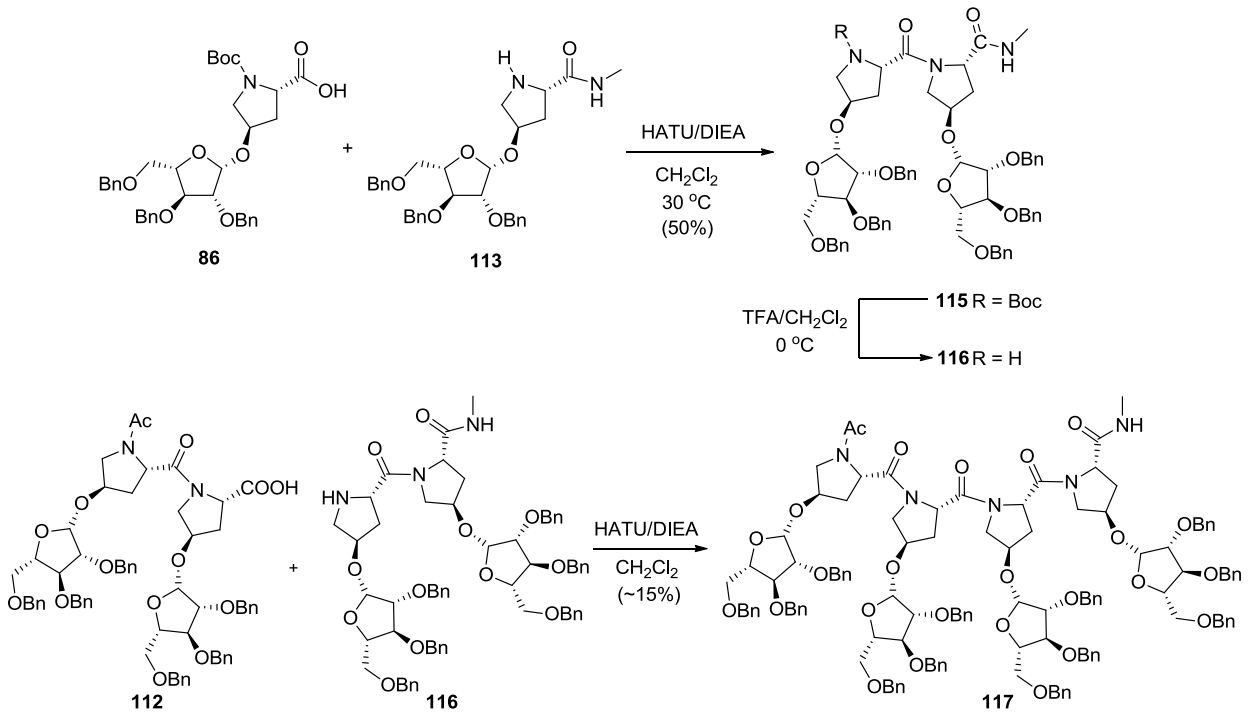
Synthesis of the end-capped triglycotripeptide began with coupling of compound **110** and **87** to give the glycodipeptide building block **111** in 60% yield (Scheme 3.27). The allyl ester of the diglycodipeptide was hydrolyzed to reveal the free acid dimer **112**. The *N*-methyl amide monomer **106** was cleaved of its Boc carbamate by TFA in dichloromethane to afford the end-capped free amine **113**. Compounds **112** and **113** were coupled under standard conditions to give the fully end-capped trimer **114** in 35% yield.



Scheme 3.27 Pre-end-capped trimer synthesis

For the synthesis of the fully end-capped tetramer, we chose again to pursue a [2+2] route. The *N*-terminus-capped dimer building block could be formed by the coupling of compounds **86** and **113** under standard conditions (Scheme 3.28). The Boc carbamate (**115**) is

subsequently removed to give the free amine **116**) which is then coupled to free acid dimer **112** with HATU to give the fully end caped tetramer product **117**.

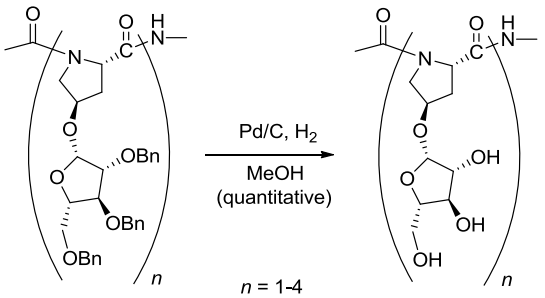


86 113 112 116 117

Scheme 3.28 Pre-end-capped tetramer synthesis

3.6 Global Deprotection

Global debenzylation of all end-capped compounds was carried out using palladium on carbon (Pd/C) and hydrogen gas in methanol (Scheme 3.29). This process gave quantitative yields for all oligomers in an overnight reaction. For the larger oligomers, higher loadings of Pd/C were useful. After filtering off the catalyst, small impurities could be washed away by dissolving the highly polar products in water and extracting with immiscible organic solvents (ethyl acetate, chloroform, dichloromethane). Lyophilization of the aqueous layer overnight afforded the end-capped glycopeptides with fully deprotected arabinoside domains as amorphous solids.

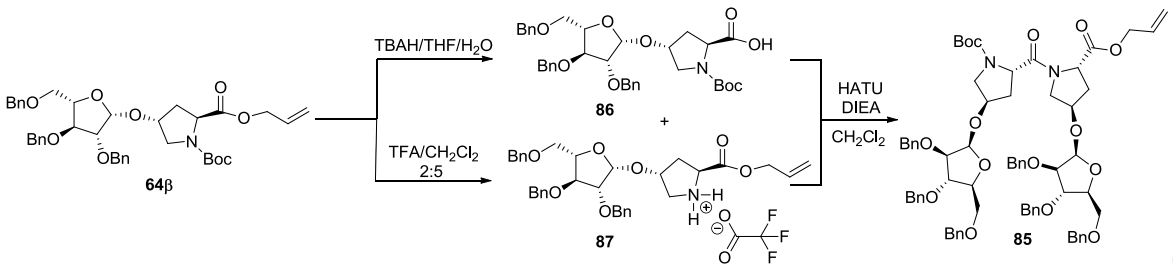


Scheme 3.29 Global debenzylation

3.7 Experimental Section

General methods: as stated in chapter 2.

3.7.1 Experimental procedures



(3.1)

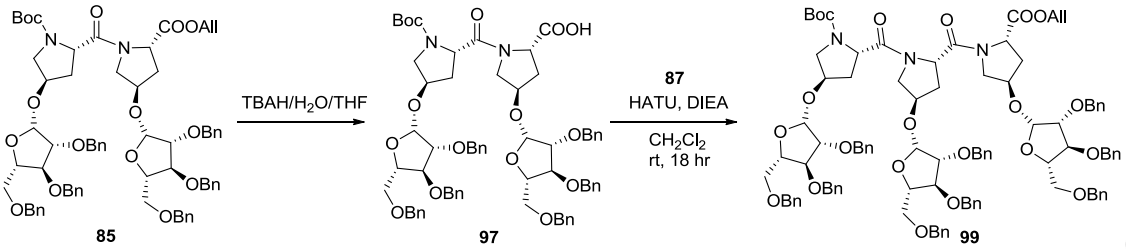
N*α*-tert-*Butyloxycarbonyl*-trans*-4-hydroxy-4-O-[2,3,5-O-Benzyl-L-arabinofuranosyl]-L-proline* (**86**) A solution of compound **64β** (662 mg, 1.0 mmol, 1.0 equiv.) in THF (8 mL) was treated with 40% aqueous tetrabutylammonium hydroxide (2.0 mL, 796 mg, 3.0 mmol, 3.0 equiv.) and stirred at RT for 1.5 h. The solvent was evaporated and the residue dissolved in EtOAc (45 mL) and washed with 1M HCl (50 mL). The aqueous portion was back extracted with EtOAc (3 x 25 mL). Organic portions were combined, filtered through $MgSO_4$, and concentrated. The crude acid **86** (quantitative) was used in the next reaction without further purification.

trans*-4-Hydroxy-4-O-[2,3,5-O-Benzyl-L-arabinofuranosyl]-L-proline Allyl Ester* (**87**). Compound **64β** (144 mg, .21 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (2.5 mL) and stirred

under N_2. The mixture was cooled to 0 °C and treated with TFA (1 mL) and thioanisole (25 µL, 27 mg, 0.21 mmol, 1.0 equiv.). The mixture was stirred at 0 °C for 30 min, warmed to RT over the next 2.5 h, and concentrated. The residue was purified by flash column chromatography, starting with 2:1 EtOAc/Hex and flushing with 4:1 CH_2Cl_2/MeOH to give **87** as a light brown oil (107 mg, 73%).

Diglycodipeptide (**85**). **87** (56 mg, .08 mmol, 1 equiv.) and **86** (62 mg, 0.10 mmol, 1.2 equiv.) were suspended in dry CH_2Cl_2 (3 mL). The mixture was cooled to 0 °C and DIEA (53 µL, 40 mg, 0.30 mmol, 3.7 equiv.) and HATU (48 mg, 0.13 mmol, 1.5 equiv.) were added successively. The reaction was heated to 30 °C while stirring under N_2 overnight. Upon completion, the mixture was diluted with CH_2Cl_2 to 25 mL total volume, washed with 1M HCl (2 x 20 mL), sat'd $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 1.5:1 Hex/EtOAc → 1.5:1 EtOAc/Hex to give title compound **85** as a light oil (58 mg, 60%). R_f 0.56 (2:1 EtOAc/Hex). $[\alpha]_D^{25}$ +30.1 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (1.30)* (s, 9H), 1.96-2.13 (m, 2H), 2.17-2.37 (m, 2H), 3.40-3.75 (m, 8H), 3.90-4.27 (m, 6H), 4.38-4.72 (m, 18H), 4.88 (5.08) (s {d, *J* = 4.0 Hz}, 1H), 4.99 (app. t, *J* = ~4.3 Hz, 1H), 5.20 (app. d, *J* = 10.4 Hz, 1H), 5.29 (app. d, *J* = 16.9 Hz, 1H), 5.82-5.91 (m, 1H), 7.25-7.33 (m, 30H); ^{13}C NMR (100 MHz, $CDCl_3$) δ *t*-Boc (28.4, 28.5)*, C-β (35.3, 35.5, 36.0, 36.6), C-δ (50.3, 51.0, 51.6, 51.7), C-α (56.6, 56.8, 57.8, 58.0), **C**H_2CH=CH_2 (65.7, 65.9), O**C**H_2Ph (72.0-72.8, 73.4, 73.5), furanose C5 73.3, C-γ (73.8, 74.3), $(CH_3)_3$**C**- (75.5, 76.8), furanose C2,3,4 (79.8-84.2), furanose C1 (98.5, 99.3, 98.9, 101.3), CH_2CH=**C**H_2 (118.4, 118.7), aromatic CH (127.7-128.6), CH_2**C**H=CH_2 (131.8, 131.9), aromatic -C- (137.4-138.2), N**C**OOR (153.7, 154.2), **C**OR (171.4, 171.6, 171.4, 171.5); HRMS (ESI) calcd $C_{70}H_{80}N_2O_{15}Na$ for $(M+Na)^+$: 1211.5456; obsd: 1211.5412.

*signals in parentheses signify pairs of rotamers.



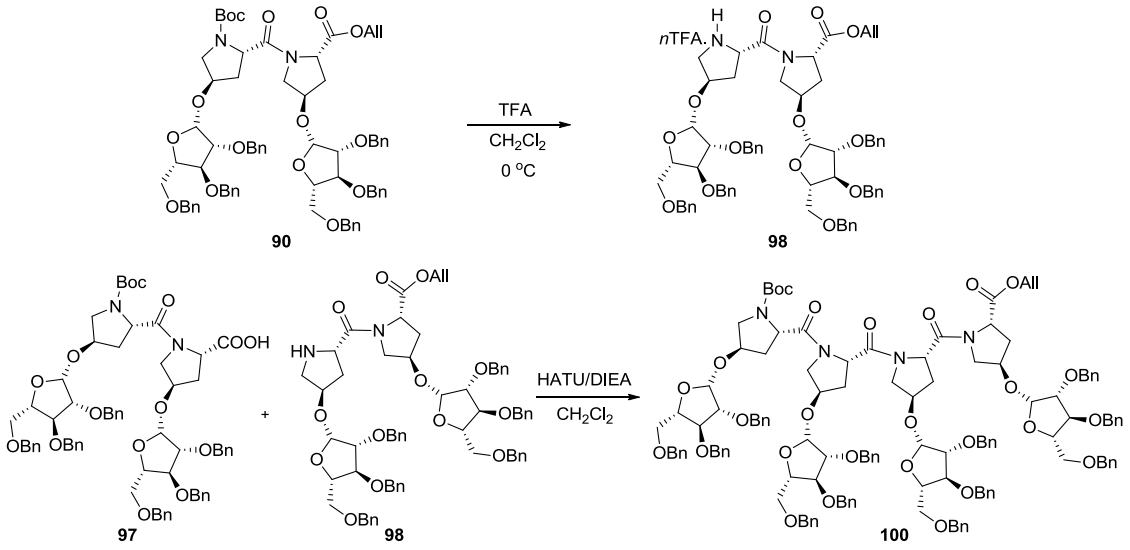
(3.2)

Dimer acid (**97**). A solution of compound **85**(180 mg, 0.15 mmol, 1.0 equiv.) in THF (3 mL) was treated with a 40% aq. solution of tetrabutylammonium hydroxide (296 µL, 160 mg, 0.45 mmol, 3.0 equiv.) and stirred at RT under N_2 for 2 h. The solvent was evaporated, the residue dissolved in EtOAc (20 mL) and washed with 1M HCl (15 mL). The aqueous layer was back-extracted with EtOAc (3 x 10 mL). The organic portions were combined, filtered through $MgSO_4$ and concentrated. The crude acid **97** was obtained in quantitative yield and submitted to the next step without further purification.

Triglycotripeptide (**99**). Compounds **87** (48 mg, 0.07 mmol, 1.0 equiv.) and **97** (81 mg, 0.07 mmol, 1.0 equiv.) were suspended in dry CH_2Cl_2 (3 mL). Diisopropylethylamine (37 µL, 30 mg, .21 mmol, 3.0 equiv.) and HATU (40 mg, 0.1 mmol, 1.5 equiv.) were added successively. The reaction mixture was stirred for 21 h under N_2. The mixture was diluted with CH_2Cl_2 to a total volume of 25 mL, washed with 1M HCl (2 x 20 mL), sat'd $NaHCO_3$ (20 mL), and brine (20 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 1.5:1 Hex/EtOAc → 1.5:1 EtOAc/Hex → 1:2 EtOAc/Hex to give the title compound **99** as a light oil (42 mg, 35%). R_f 0.62 (2:1 EtOAc/Hex). $[\alpha]_D^{25}$ 42.6 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (1.33)* (s, 9H), 1.75-1.80 (m, 1H), 1.90-2.00 (m, 1H), 2.04-2.34 (m, 4H), 3.06-3.67 (m, 8H), 3.84-4.13 (m, 12H), 4.38-4.72 (m, 27H), 4.84 (d, *J* = 4.2 Hz, 0.5H), 4.87 (d, *J* = 4.1Hz, 0.5H), 4.92 (d, *J* = 3.8, 0.5H), 5.09 (d, *J* = 2.8 Hz, 0.5H), 5.15 (d, *J* = 2.1 Hz, 0.5H), 5.16 (d, *J* = 4.0 Hz, 0.5H), 5.19-5.30 (m, 2H), 5.81-5.91 (m, 1H), 7.28-7.33 (m, 45H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.5 (*t*-Boc CH_3), (34.9, 35.1,

35.5, 35.7, 36.0, 36.3) (C-β), (50.0, 50.1, 50.5, 50.7, 51.6, 51.9) (C-δ), (56.6, 56.8, 56.9, 57.0, 57.9, 58.1) (C-α), 65.7 (**C**H_2CH=CH_2), (72.0-72.7) (O**C**H_2Ph), (73.1, 73.3) (furanose C5), (73.61, 73.7, 74.5) (C-γ), (79.6, 79.7) (Boc $(CH_3)_3$**C**-), (79.9-84.2) (furanose C2,3,4), (98.2, 98.6, 98.9, 101.0) (furanose C1), 118.4 (CH_2CH=**C**H_2), (127.8-128.4) (Ar 3° CH), 131.8 (CH_2**C**H=CH_2), (137.5-138.2) (Ar 4° -C-), (153.8, 154.2) (N**C**OO^{t}Bu), (170.8, 170.8, 171.0, 171.1, 171.4, 171.5) (**C**OR); HRMS (ESI) calcd $C_{101}H_{112}N_3O_{21}Na$ for $(M+Na)^+$: 1726.7759; obsd: 1726.7750.

*Signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond



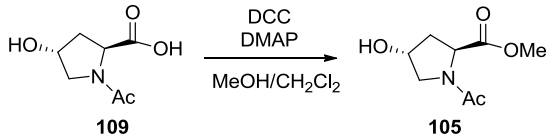
(3.3)

Dimer amine **98**. Diglycodipeptide **85** (168 mg, .14 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (3 mL) and stirred under N_2. The mixture was cooled to 0 °C and treated with TFA (1.5 mL). The mixture was stirred at 0 °C for 3 h and concentrated to give compound **98** in quantitative yield. The residue was submitted to the next step without further purification.

Tetraglycotetrapeptide **100**. Compounds **97** (145 mg, 0.13 mmol, 1 equiv.) and **98** (170 mg, 0.14 mmol, 1.1 equiv.) were suspended in dry CH_2Cl_2 (5 mL). HATU (50 mg, 0.13 mmol, 1.0 equiv.) and DIEA (110 µL, 82 mg, 0.63 mmol, 5.0 equiv.) were added successively. The mixture

was stirred for 18 h under N_2. Upon completion, the solvent was evaporated and the residue diluted with EtoAc (30 mL), washed with 1M HCl (30 mL), sat'd $NaHCO_3$ (30 mL), and brine (30 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 1.5:1 Hex/EtOAc → 1:1 Hex/EtOAc → 1:1.5 Hex/EtOAc → 1:2 Hex/EtOAc to give the title compound **100** as a light oil (138 mg, 49%). R_f 0.80 (2:1 EtOAc/Hex). $[\alpha]_D^{25}$ +42.2 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.30 [1.26]* (s, 9H), 1.70-2.30 (m, 8H), 3.08-3.66 (m, 16H), 3.76-4.20 (m, 15H), 4.23-4.67 (m, 31H), 4.76-5.10 (anomeric signals, 4H), 5.18 (d, *J* = 10.4 Hz, 1H), 5.27 (d, *J* = 17.3 Hz, 1H), 5.78-5.88 (m, 1H), 7.26-7.31 (m, 60H); ^{13}C NMR (100 MHz, $CDCl_3$) δ (28.5, 29.7) (Boc CH_3), (34.8, 35.0, 35.1, 35.3, 35.4, 35.9, 36.2) (C-β), (50.0, 50.1, 50.2, 50.4 50.5, 50.8, 51.6, 52.1) (C-δ), (56.7, 56.8, 57.0, 57.1, 57.2, 57.9) (C-α), (65.8) (**C**H_2CH=CH_2), (72.0-72.6) (O**C**H_2Ph), (73.2, 73.3, 73.4) (furanose C5), (73.8, 74.0, 74.1, 74.3) (C-γ), (79.6, 79.7) (Boc $(CH_3)_3$**C**-), (79.9-84.3) (furanose C2,3,4), (98.0, 98.1, 98.3, 98.6, 98.9, 99.2, 99.4, 100.8) (furanose C1), (118.5, 118.6) (CH_2CH=**C**H_2), (127.8-128.7) (Ar CH), 131.8 (CH_2**C**H=CH_2), (137.5-138.2) (Ar -C-), (153.9, 154.2) (N**C**OO^tBu), (170.4, 170.5, 170.7, 170.9, 171.4) (**C**OR); HRMS (ESI) calcd $C_{132}H_{145}N_4O_{27}Na$ for $(M+Na)^+$: 2242.0067; obsd: 2243.0072.

*signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond.

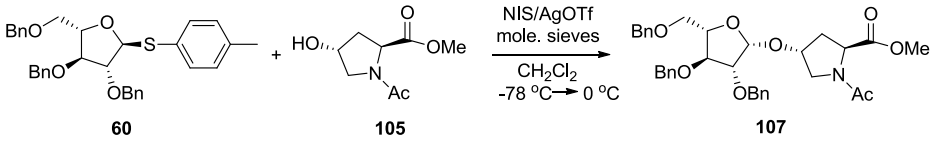


109 **105** (3.4)

N-*Acety-L*-trans-*4-hydroxy-L-proline methyl ester* (**105**). Dicyclohexylcarbodiimide (119 mg, 0.58 mmol, 1.0 equiv.) and 4-DMAP (18 mg, 0.15 mmol, 0.25 equiv.) were added sequentially to a suspension of Ac-Hyp-OH (**109**) (100 mg, 0.58 mmol, 1.0 equiv.) in dry MeOH (2 mL) and CH_2Cl_2 (2 mL). The mixture was stirred overnight under N_2 after which the solvent was

evaporated. The residue was triturated with CH_2Cl_2 and filtered to remove dicyclohexylurea. The filtrate was concentrated and purified by flash column chromatography, eluting with CH_2Cl_2/MeOH (14:1 → 10:1) to give compound **105** as an amorphous solid (71 mg, 66%). R_f 0.33 (10:1 CH_2Cl_2/MeOH). $[\alpha]_D^{25}$ -89.9 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.03-2.10 (2.15-2.23) (m, 1H), 2.07 (1.96)* (s, 3H), 2.26-2.32 (2.41-2.47) (m, 1H), 3.51 (app. d, *J* = 11.2 Hz, 1H), 3.72 (3.77) (s, 3H), 3.74-3.79 (m, 1H), 4.52-4.57 (4.45) (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.2 (21.6), 38.0 (39.7), 52.3 (52.7), 55.9 (54.5), 57.5 (58.8), 70.1 (68.5), 170.0 (170.7), 173.0 (172.7); HRMS (ESI) calcd for $C_8H_{14}NO_4$ $(M+H)^+$: 188.0917, obsdd: 188.0919.

*signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond.

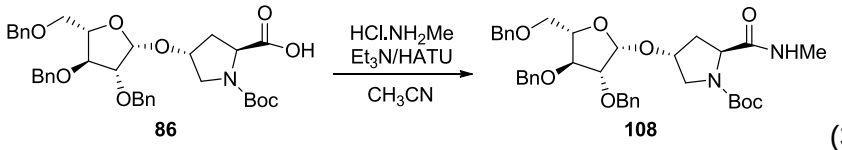


(3.5)

N-*Acetyl*-trans-*4-hydroxy-4-O-[2,3,5-O-benzyl-L-arabinofuranosyl]-L-proline Methyl Ester***.** A solution of compounds **60** (342 mg, 0.65 mmol, 1.0 equiv.) and **105** (124 mg, 0.66 mmol, 1.0 equiv.) in dry CH_2Cl_2 (40 mL) was stirred with activated powdered 4Å molecular sieves (1.0 g) under N_2 for ~30 min at RT. The suspension was cooled to -78 °C (acetone/dry ice) and then NIS (231 mg, 1.0 mmol, 1.5 equiv.) and AgOTf (83 mg, 0.32 mmol, 0.5 equiv.) were added. The reaction was allowed to gradually reach 0 °C over 1.5 h, at which time it was quenched with Et_3N (2 mL) and filtered. The filtrate was diluted with EtOAc (50 mL) and washed with 10% aqueous $Na_2S_2O_3$ (50 mL) and brine (50 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue, determined to be a 3:1 β:α ratio by NMR, was purified by column chromatography, eluting with 3:1 Hex/EtOAc to afford **107** (317 mg, 83%) as an orange oil. R_f 0.34 (8:1 EtOAc/Hex). $[\alpha]_D^{25}$ 39.2 (*c* 0.5, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02-2.08 (2.10-2.17)* (m, 1H), 2.03 (1.84) (s, 3H), 2.31-2.40 (m, 1H), 3.41 (dd, *J* = 10.6, 3.6 Hz, 1H),

3.49-3.52 (m, 2H), 3.71 (3.75) (s, 3H), 3.72-3.74 (m, 1H), 4.07-4.14 (m, 3H), 4.29-4.43 (m, 1H), 4.48-4.73 (m, 7H), 4.90 (4.98) (d, *J* = 3.6 (4.0) Hz, 1H), 7.27-7.36 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.3 (21.5), 36.0 (38.0), 52.3 (52.7), 52.8 (50.6), 57.5 (58.8), 71.9 (72.2), 72.5 (72.4), 72.7 (73.1), 73.4 (73.3), 76.3, 80.1, 82.4, 84.2 (83.9), 100.5 (99.1), 127.8, 127.9, 128.0, 128.1 (2c), 128.4, 128.5, 128.6, 137.6, 137.8 (137.9), 138.1 (2c), 169.3, 172.7 (172.6); HRMS (ESI) calcd for $C_{34}H_{40}NO_8$ $(M+H)^+$: 590.2748, obsd: 590.2758.

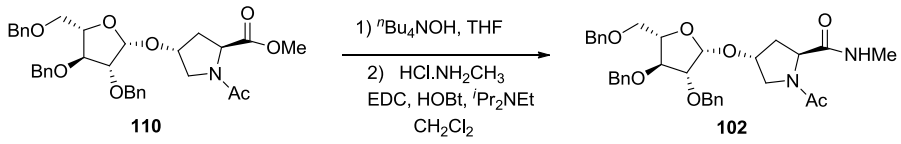
*signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond



(3.6)

HATU (50 mg, 0.13 mmol, 1.5 equiv) and triethylamine (62 µL, 45 mg, 0.45 mmol, 5 equiv.) were added to a solution of compounds **86** (56 mg, 0.09 mmol, 1 equiv.) and methylamine hydrochloride (12 mg, 0.18 mmol, 2 equiv.) in acetonitrile under an atmosphere of N_2. The mixture was stirred for 18 h and the solvent evaporated. The residue was diluted with EtOAc (30 mL), washed with 1 M HCl (30 mL) and aq. $NaHCO_3$ (30 mL), filtered through $MgSO_4$, and concentrated. The residue was purified by flash column chromatography, eluting with 8:1 EtOAc/Hex to give the amide product **108** as a light oil (40 mg, 70%). R_f 0.32 (8:1 EtOAc/Hex). $[\alpha]_D^{25}$ 19.6 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (1.38)* (s, 9H), 2.08 (br s, 1H), 2.43 (2.32) (br s, 1H), 2.78 (2.77) (s, 3H), 3.43-3.47 (3.75-3.78) (m, 2H), 3..52 (app d, *J* = 3.3 Hz, 2H), 4.06-4.31 (m, 5H), 4.50-4.70 (m, 6H), 4.97 (s, 1H), 6.57 (5.74) (s, 1H), 7.26-7.36 (m, 15H); HRMS (ESI) calcd for $C_{37}H_{46}N_2O_8$ $(M+H)^+$: 647.3327, obsd: 647.3323.

*signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond.



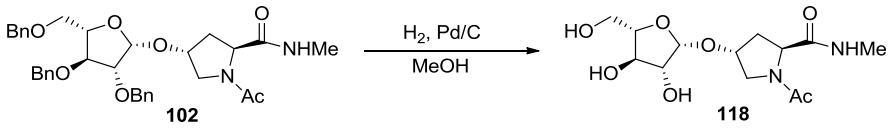
(3.7)

N-*Acetyl*-trans-*4-hydroxy-4-O-[2,3,5-O-benzyl-L-arabinofuranosyl]-L-proline Methyl Amide* (**102**). A solution of compound **110** (121 mg, 0.21 mmol, 1.0 equiv.) in dry THF (4 mL) was treated with a 40% aq. solution of tetrabutylammonium hydroxide (401 µL, 160 mg, 0.62 mmol, 3.0 equiv.) and stirred at RT under N_2 for 1.5 h. The solvent was evaporated and the residue dissolved in EtOAc (25 mL), washed with 1M HCl (25 mL). The aqueous layer was back-extracted with EtOAc (10 mL). The organic portions were combined, filtered through $MgSO_4$ and concentrated. The crude acid was obtained in quantitative yield and submitted to next step without further purification.

A suspension of the free acid (56 mg, 0.10 mmol, 1.0 equiv.) and methylamine hydrochloride (8 mg, 0.10 mmol, 1.0 equiv.) in dry CH_2Cl_2 was cooled to 0 °C while stirring under N_2. Diisopropylethylamine (19 µL, 14 mg, 0.11 mmol, 1.1 equiv.) was added, followed by EDC (21 mg, 0.11 mmol, 1.1 equiv.) and HOBt (17 mg, 0.13 mmol, 1.3 equiv.). The ice bath was then removed and the reaction was left to stir overnight. The mixture was diluted with CH_2Cl_2 (25 mL) and washed with 1 M HCl (25 mL), sat'd $NaHCO_3$ (aq.) (25 mL), and brine (25 mL). The organic layer was filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 19:1 CH_2Cl_2/MeOH to give compound **102** (35 mg, 61%) as an oil. R_f 0.49 (10:1 CH_2Cl_2/MeOH). $[\alpha]_D^{25}$ 20.6 (*c* 1.0, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02 (1.84)* (s, 3H), 2.04-2.07 (m, 1H), 2.53 (dt, *J* = 13.0, 5.1 Hz, 1H), 2.72 (2.77) (d, *J* = 4.8 Hz, 3H), 3.36 (3.43) (dd, *J* = 11.6 (12.7), 4.6 (4.1) Hz, 1H), 3.53-3.56 (m, 2H), 3.61 (dd, *J* = 10.7, 5.8 Hz, 1H), 4.07-4.11 (m, 3H), 4.39-4.44 (app. p, *J* = 5.4 Hz, 1H), 4.48-4.73 (m, 7H), 4.94 (4.99) (d, *J* = 3.8 Hz, 1H), 7.26-7.35 (m, 15H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.5, 26.2, 34.2, 52.9, 58.37, 72.1, 72.5, 72.7, 73.3, 76.3, 80.1, 82.7, 84.2, 127.7, 127.9, 128.0, 128.1, 128.4,

128.5, 128.5, 137.6, 137.9, 138.1, 170.5, 171.4; HRMS (ESI) calcd for $C_{34}H_{41}N_2O_7$ $(M+H)^+$: 589.2908, obsd: 589.2913.

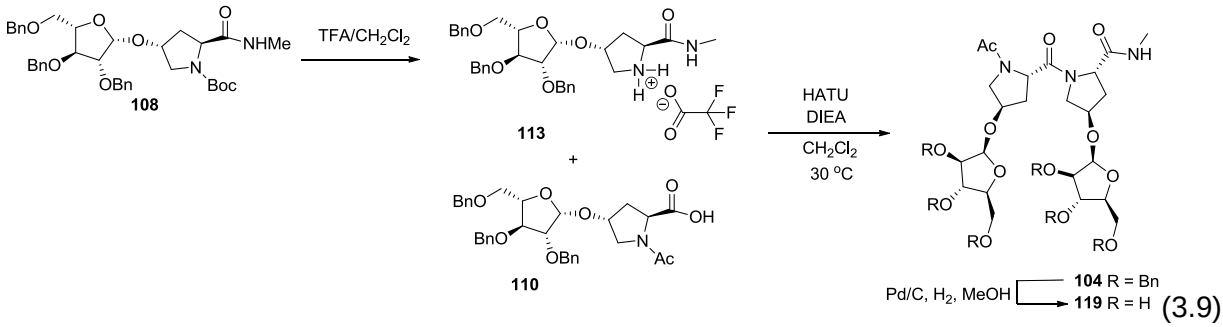
*Signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond. Interestingly, the ^{13}C spectrum only displayed signals for one species.



(3.8)

N-*Acetyl-4-O-[L-arabinofuranosyl]*-trans-*4-hydroxy-L-proline Methyl Amide* (**118**). Palladium on carbon (10% w/w, 45 mg) was added to a solution of compound **102** (35 mg, 0.06 mmol) in MeOH (2 mL). The suspension was stirred under an atmosphere of H_2 gas for 18 h. Upon completion, the mixture was filtered through Celite® and concentrated to give the triol **118** (19 mg, quantitative). $[\alpha]_D^{25}$ 25.6 (*c* 0.5, MeOH). ^1H NMR (400 MHz, CD_3OD) δ 2.08 (1.93)* (s, 3H), 2.03-2.10 (2.13-2.19) (m, 1H), 2.47-2.53 (2.59-2.64) (m, 1H), 2.73 (2.77) (s, 3H), 3.34 (s, 1H), 3.56 (dd, *J* = 11.6, 7.1 Hz, 1H), 3.68-3.78 (m, 3H), 3.85-3.91 (m, 1H), 3.96 (dd, *J* = 7.8, 4.6 Hz, 1H), 4.41 (4.51) (t, *J* = 8.0 (7.7) Hz, 1H), 4.45-4.47 (m, 1H), 4.99 (4.95) (d, *J* = 4.6 (4.5) Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 21.0 (20.2), 25.0 (25.1), 36.5 (38.4), 53.5 (51.7), 59.0 (60.1), 63.9 (63.8), 75.0 (74.4), 76.4, 77.2, 83.0, 101.2 (100.7), 171.2 (171.6), 173.7 (173.5); HRMS (ESI) calcd for $C_{13}H_{23}N_2O_7$ $(M+H)^+$: 319.1500, obsd: 319.1486.

*Signals in parentheses refer to the minor conformation arising from restricted rotation about the prolyl peptide bond.



(3.9)

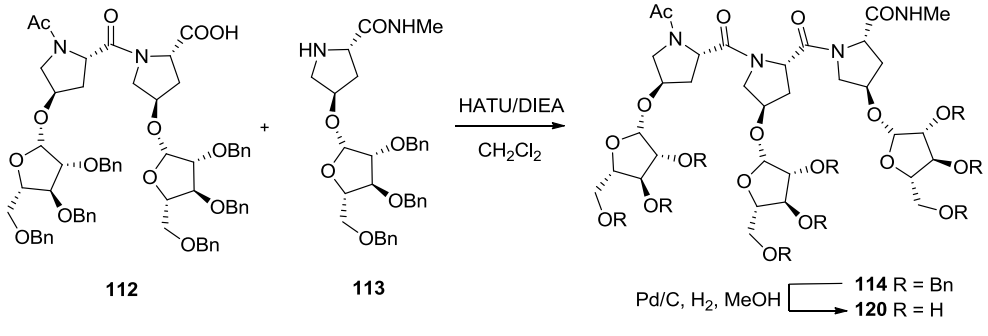
Fully deprotected diglycodipeptide **104**. The methyl amide **108** (199 mg, 0.31 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (5 mL) and cooled to 0 ^oC under an atmosphere of N_2. Trifluoroacetic acid (1.6 mL) was then added and the mixture stirred for 3 h at 0 ^oC and concentrated. The free amine was submitted to the next reaction without further purification.

The acid **110** (94 mg, 0.14 mmol, 1 equiv.) and amine **113** (104 mg, 0.14 mmol, 1 equiv.) were dissolved in dry CH_2Cl_2 and stirred under N_2. The coupling reagent HATU (93 mg, 0.21 mmol, 1.5 equiv.) was added and the reaction stirred for 15 min, after which DIEA (102 mg, 137 µL, 0.79 mmol, 5.5 equiv.) was added to the mixture. The reaction was (mildly) heated to 30 ^oC and stirred for 18 h. The mixture was diluted with CH_2Cl_2 (25 mL), washed with 1 M HCl (25 mL) and brine (25 mL), filtered through $MgSO_4$, and concentrated. The residue was purified by flash column chromatography, eluting with 14:1 CH_2Cl_2/MeOH to give the dimer **104** as a cloudy oil (86 mg, 55%).

Palladium on carbon (20 mg, 10% w/w) was added to a solution of compound **104** (19 mg, 0.018 mmol) in MeOH (1 mL). The suspension was stirred under an atmosphere of H_2 gas for 18 h. Upon completion, the mixture was filtered through Celite® washing well with MeOH, and concentrated. The residue was dissolved in H_2O (10 mL) and washed with CH_2Cl_2 (3 x 10mL) to remove organic impurities. The aqueous layer was lyophilized to give the fully deprotected dimer **119** (10 mg, quantitative) as an amorphous solid. $[\alpha]_D^{25}$ +30.8 (*c* 0.5, MeOH). ^1H NMR (400 MHz, CD_3OD) δ 2.00-2.11 (m, 2H)*, 2.06 (s, 3H), 2.44-2.49 (m, 1H), 2.58-2.63 (m, 1H), 2.73 (s, 3H), 3.55-3.61(m, 2H), 3.69-3.78 (m, 7H), 3.89-3.99 (m, 4H), 4.12 (d, *J* = 11.1 Hz, 1H),

4.46 (t, *J* = 8.1 Hz, 1H), 4.52 (br s, 1H), 4.76 (t, *J* = 8.1 Hz, 1H), 5.01 (d, *J* = 4.2 Hz, 1H), 5.04 (d, *J* = 4.4 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 20.8, 25.0, 35.6, 36.1, 52.4, 53.5, 56.9, 59.4, 74.9, 75.2, 76.2, 76.7, 77.3, 77.4, 83.0, 83.1, 100.9, 101.0, 170.7, 171.9, 173.4; HRMS (ESI+) calcd for $C_{23}H_{38}N_3O_{13}$ $(M+H)^+$: 564.2399, obsd: 564.2390.

*Product is predominantly a single species in solution thus minor rotamer not reported.



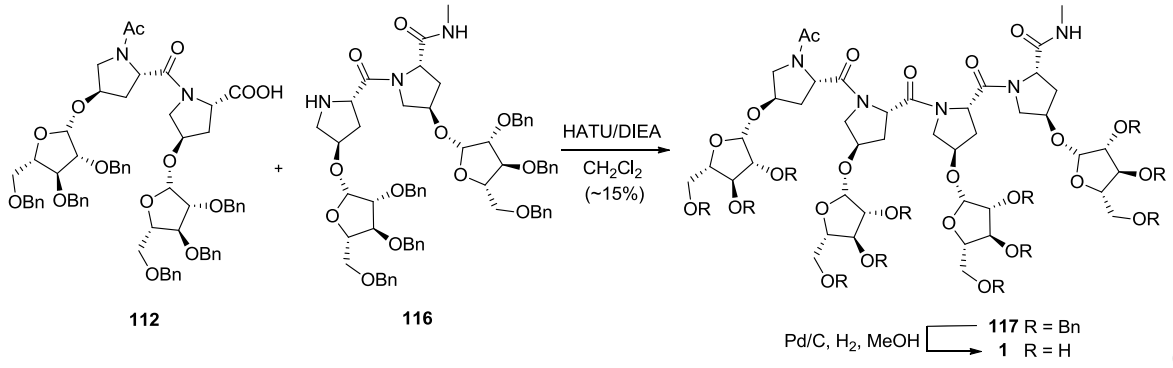
(3.10)

Fully deprotected triglycotripeptide **120**. The acid **112** (156 mg, 0.14 mmol, 1.0 equiv.) and amine **113** (94 mg, 0.14 mmol, 1.0 equiv.) were dissolved in dry CH_2Cl_2 (5 mL) and stirred under N_2. The coupling reagent HATU (82 mg, 0.22 mmol, 1.5 equiv.) was added and the reaction stirred for 15 min, after which DIEA (124 µL, 92 mg, 0.71 mmol, 5.0 equiv.) was added to the mixture. The reaction was stirred at rt for 21 h. The mixture was diluted with EtOAc (25 mL), washed with 1 M HCl (25 mL), sat'd $NaHCO_3$ (25 mL) and brine (25 mL), filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 19:1 CH_2Cl_2/MeOH to give the protected trimer **114** as a light oil (81 mg, 35%).

Palladium on carbon (100 mg, 10% w/w) was added to a solution of compound **114** (11 mg, 6.8 µmol) in MeOH (1.5 mL). The suspension was stirred under an atmosphere of H_2 gas for 24 h. The mixture was filtered through Celite®, washing well with MeOH, and concentrated. The residue was dissolved in H_2O (10 mL) and washed with CH_2Cl_2 (3 x 10 mL) to remove organic impurities. The aqueous layer was lyophilized to give the fully deprotected trimer **120** in quantitative yield (5.5 mg). $[\alpha]_D^{25}$ -17.4 (*c* 0.1, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 2.01-2.17

(m, 3H), 2.11 (s, 3H), 2.47-2.66 (m, 3H), 2.75 (s, 3H), 3.58-3.66 (m, 3H), 3.69-3.78 (m, 10H), 3.86-4.01 (m, 6H), 4.15 (d, *J* = 11.5 Hz, 1H), 4.26 (d, *J* = 11.1 Hz, 1H), 4.48 (t, *J* = 8.5 Hz, 2H), 4.53 (br s, 2H), 4.60 (br s, 1H), 4.79 (t, *J* = 8.3 Hz, 1H), 5.02 (d, *J* = 4.4 Hz, 1H), 5.04 (app t, *J* = 4.8 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 24.1, 28.6, 37.5, 37.7, 38.5, 55.4, 55.5, 56.4, 59.6, 60.2, 62.4, 63.2, 65.9, 65.9(5), 66.0, 72.5, 74.4, 77.1, 77.1(5), 77.2, 78.8, 78.8(2), 78.9, 79.0, 79.0(3), 79.3, 84.6, 84.6(7), 84.7, 102.7, 102.8, 102.9, 173.8, 174.6, 174.8, 175.5, 176.5; HRMS (ESI+) calcd for $C_{33}H_{52}N_4O_{19}$ $(M+H)^+$: 809.3299, obsd: 809.3314.

*Product is predominantly a single species in solution thus minor rotamer not reported.



(3.11)

Fully deprotected tetraglycotetrapeptide **1**. The acid **112** (47 mg, 0.04 mmol, 1.0 equiv.) and amine **116** (71 mg, 0.06 mmol, 1.4 equiv.) were dissolved in dry CH_2Cl_2 (1.75 mL) and stirred under N_2. The coupling reagent HATU (17 mg, 0.04 mmol, 1.0 equiv.) was added and the reaction stirred for 15 min, after which DIEA (124 µL, 92 mg, 0.16 mmol, 4.0 equiv.) was added to the mixture. The reaction was stirred at rt for 20 h. The mixture was diluted with EtOAc (25 mL), washed with 1 M HCl (25 mL) and brine (25 mL), filtered through $MgSO_4$ and concentrated. The residue was purified by flash column chromatography, eluting with 19:1 CH_2Cl_2/MeOH to give the protected tetramer **117** as a light oil (14 mg, 15%).

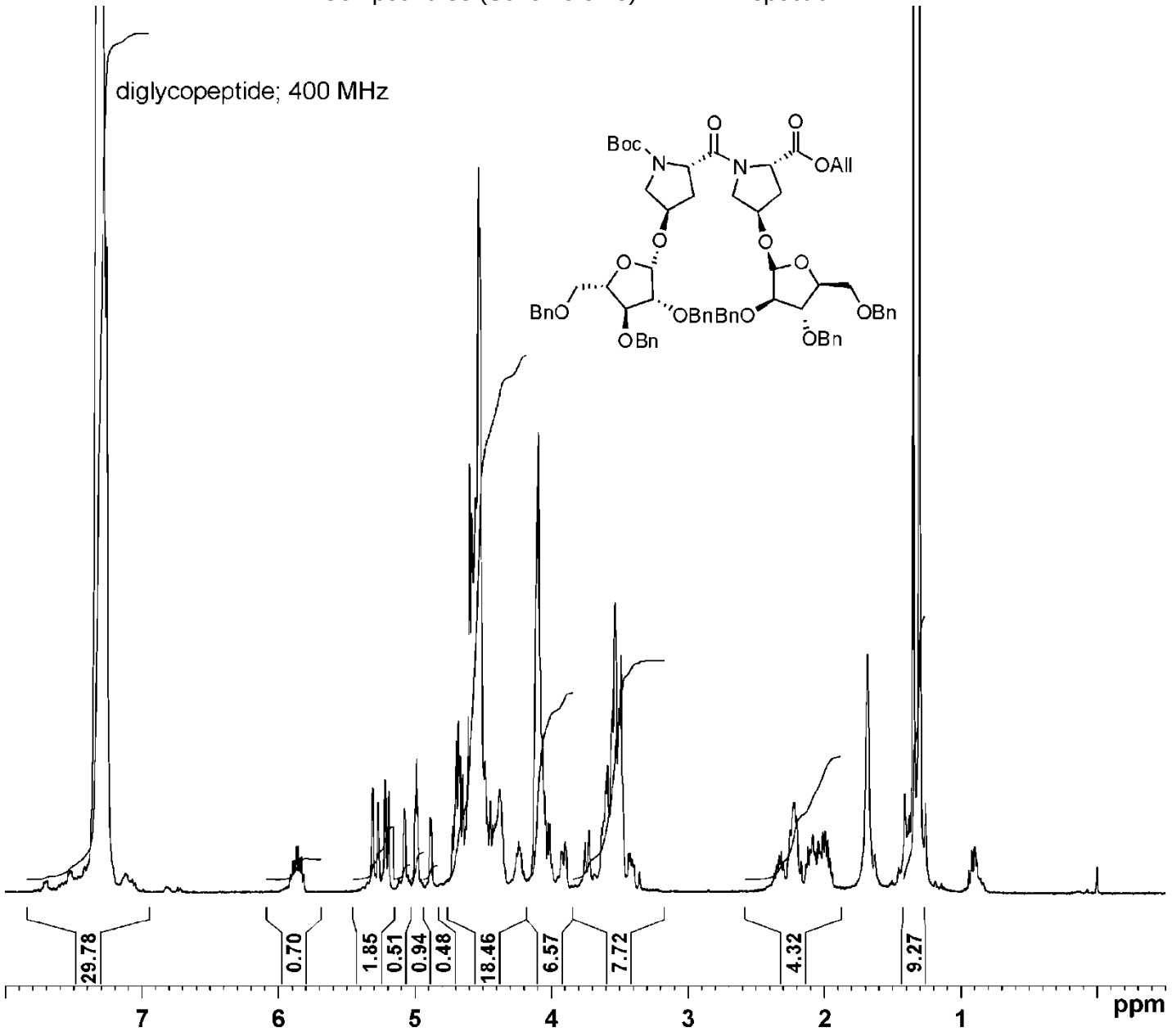
Palladium on carbon (25 mg, 10% w/w) was added to a solution of compound **117** (3 mg, 1.4 µmol) in MeOH (0.6 mL). The suspension was stirred under an atmosphere of H_2 gas for 24 h.

The mixture was filtered through Celite®, washing well with MeOH, and concentrated. The residue was dissolved in H_2O (10 mL) and washed with CH_2Cl_2 (3 x 10mL) to remove organic impurities. The aqueous layer was lyophilized to give the fully deprotected tetramer **1** in quantitative yield (1.5 mg). $[\alpha]_D^{25}$ +60.8 (*c* 0.075, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 2.04-2.19 (m, 4H), 2.09 (s, 3H), 2.47-2.67 (m, 4H), 2.76 (s, 3H), 3.59-3.65 (m, 4H), 3.73-3.79 (m, 12H), 3.86-4.01 (m, 8H), 4.15 (app d, *J* = 11.5 Hz, 3H), 4.50 (t, *J* = 8.0 Hz, 4H), 4.56 (br s, 4H), 5.04 (d, *J* = 4.5 Hz, 2H), 5.04 (d, *J* = 4.3 Hz, 4H); HRMS (ESI+) calcd for $C_{43}H_{67}N_5O_{25}Na$ $(M+Na)^+$: 1076.4017, obsd: 1076.3992.

*Product is predominantly a single species in solution thus minor rotamer not reported.

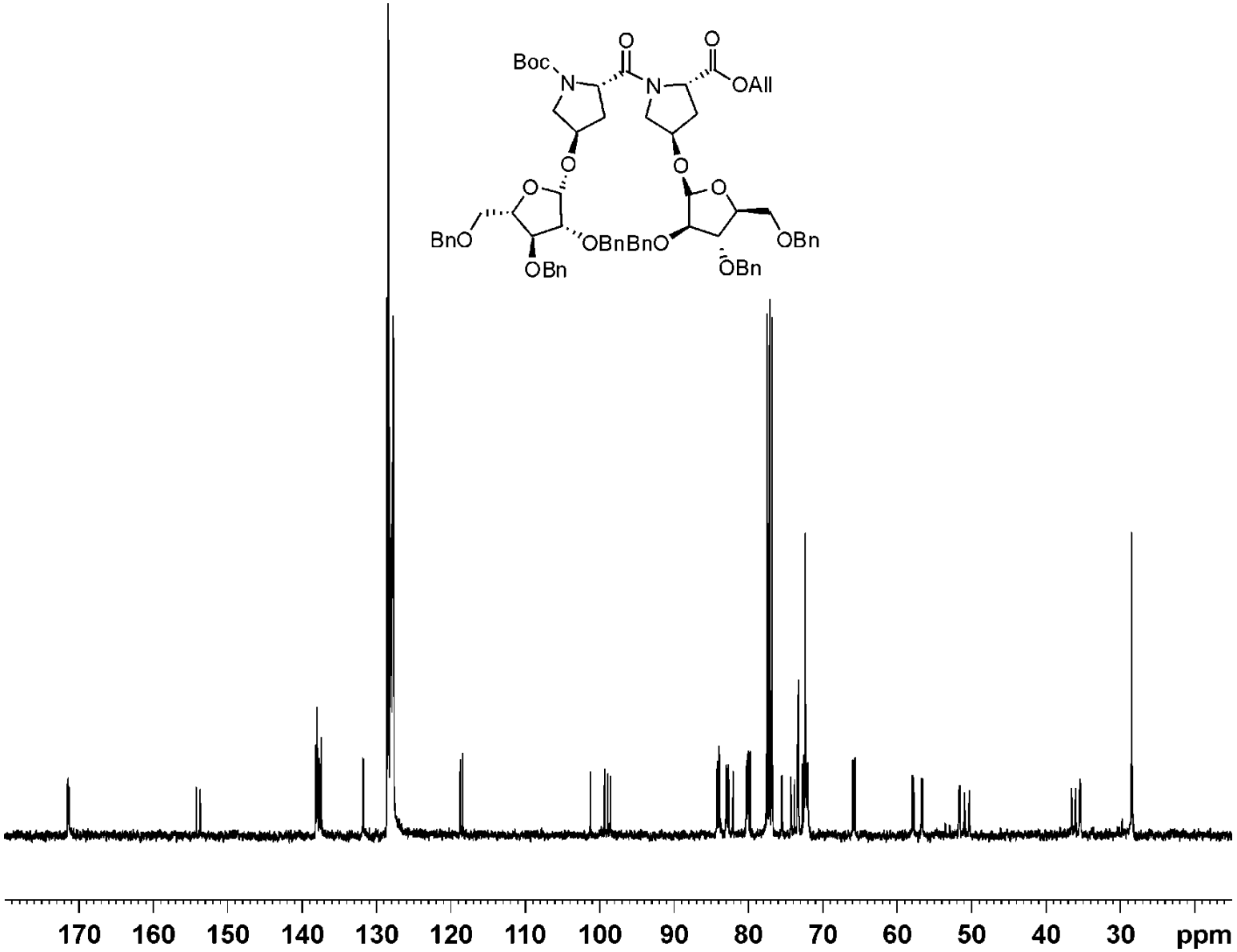
3.7.2 Spectra

Compound **85** (Scheme 3.15) - ^{1}H NMR spectrum

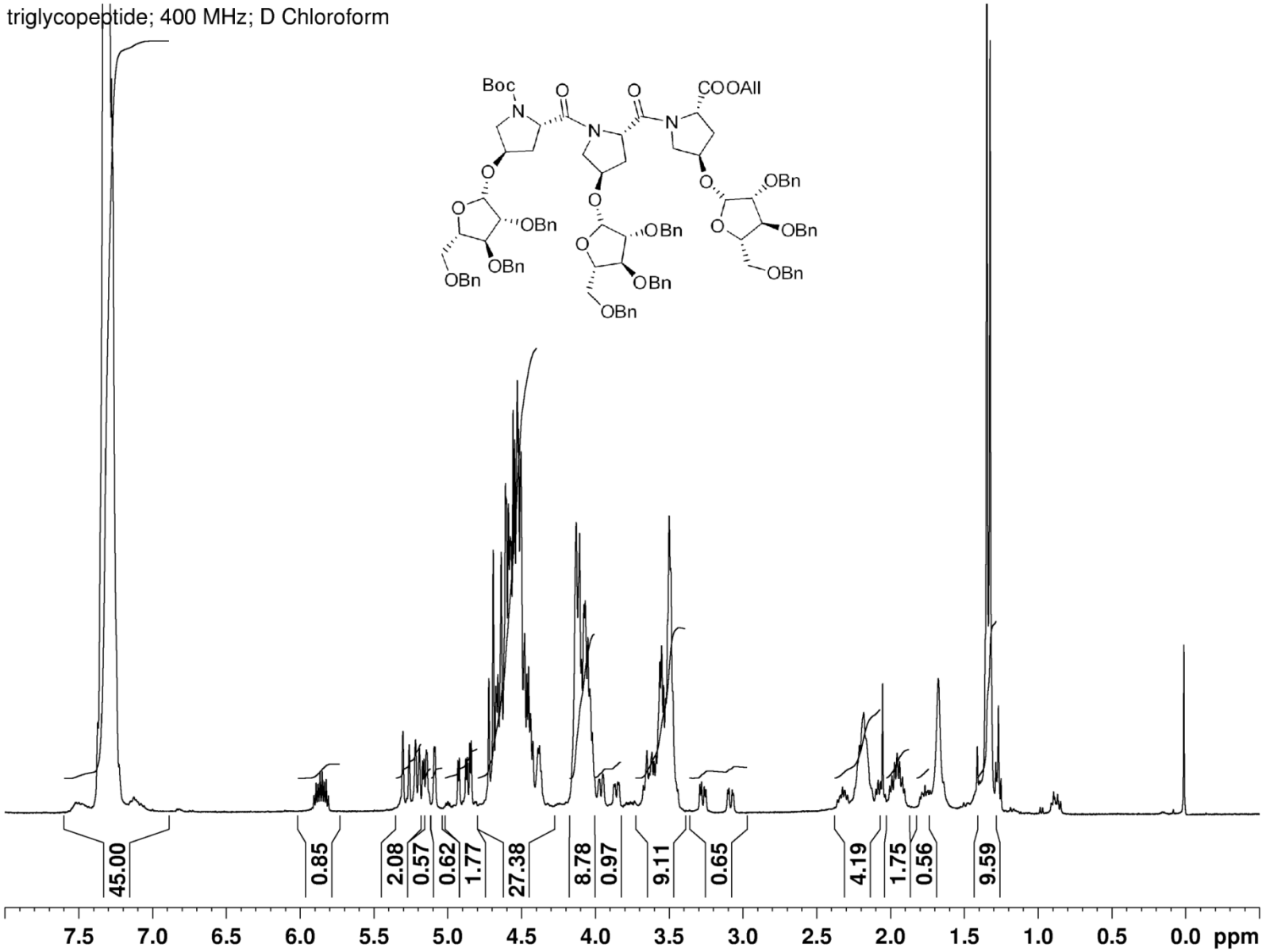


Compound **85** (Scheme 3.15) – ^{13}C NMR spectrum

diglycopeptide; 400 MHz; D Chloroform

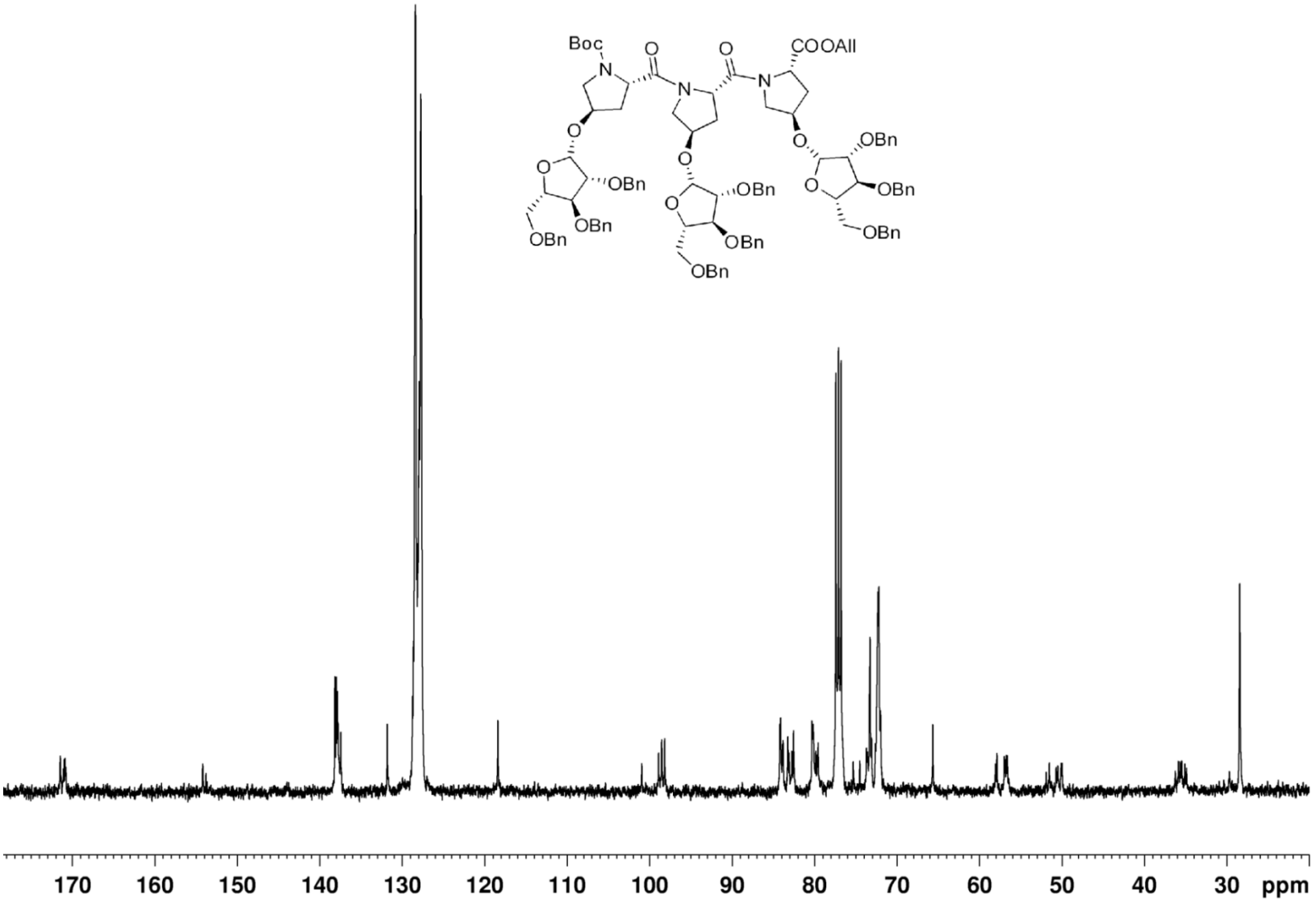


Compound **99** (Scheme 3.18) - ^{1}H NMR spectrum

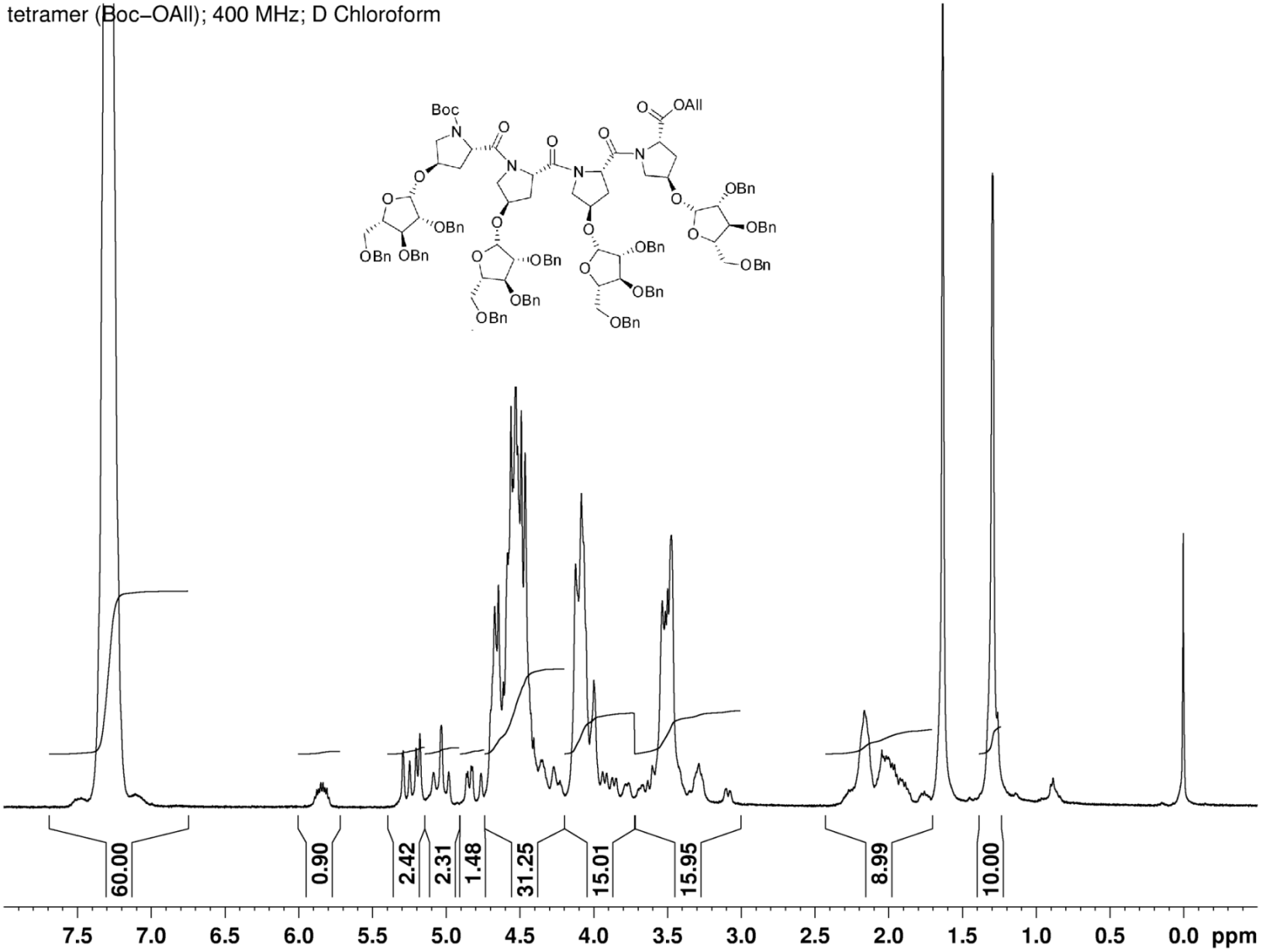


Compound **99** (Scheme 3.18) – ^{13}C NMR spectrum

Triglycopeptide; 400 MHz; D Chloroform

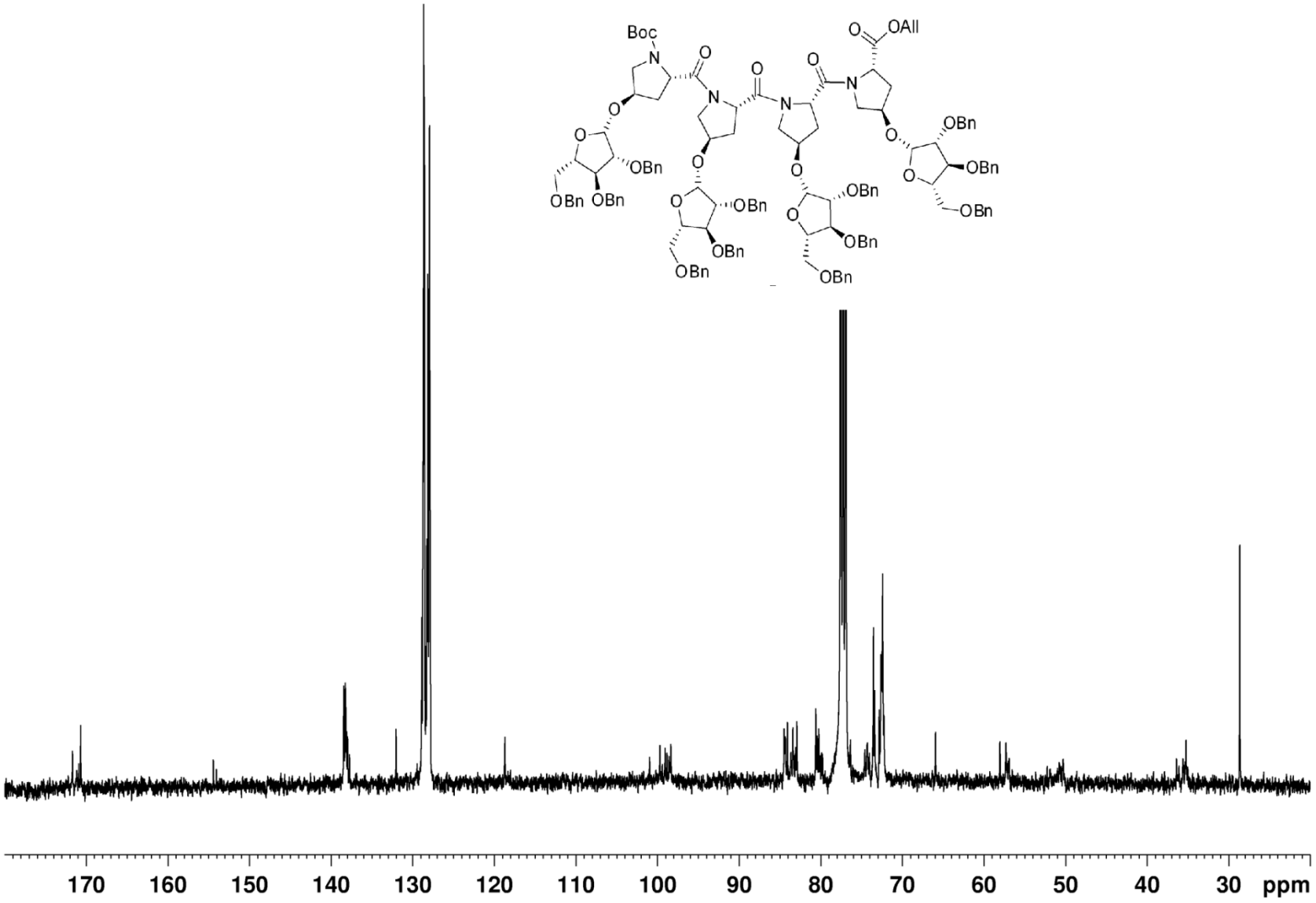


Compound **100** (Scheme 3.19) - ^{1}H NMR spectrum

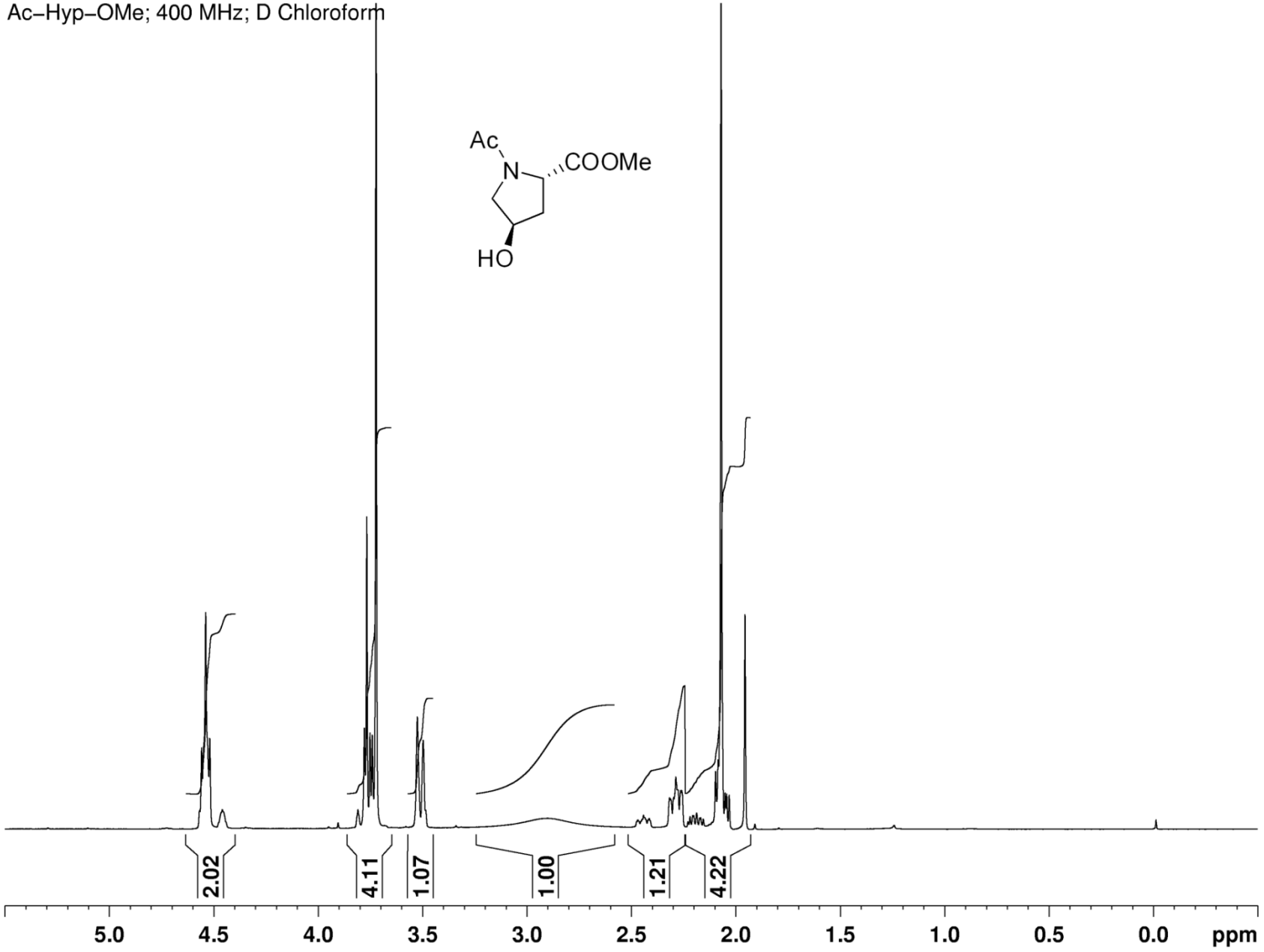


Compound **100** (Scheme 3.19) – ^{13}C NMR spectrum

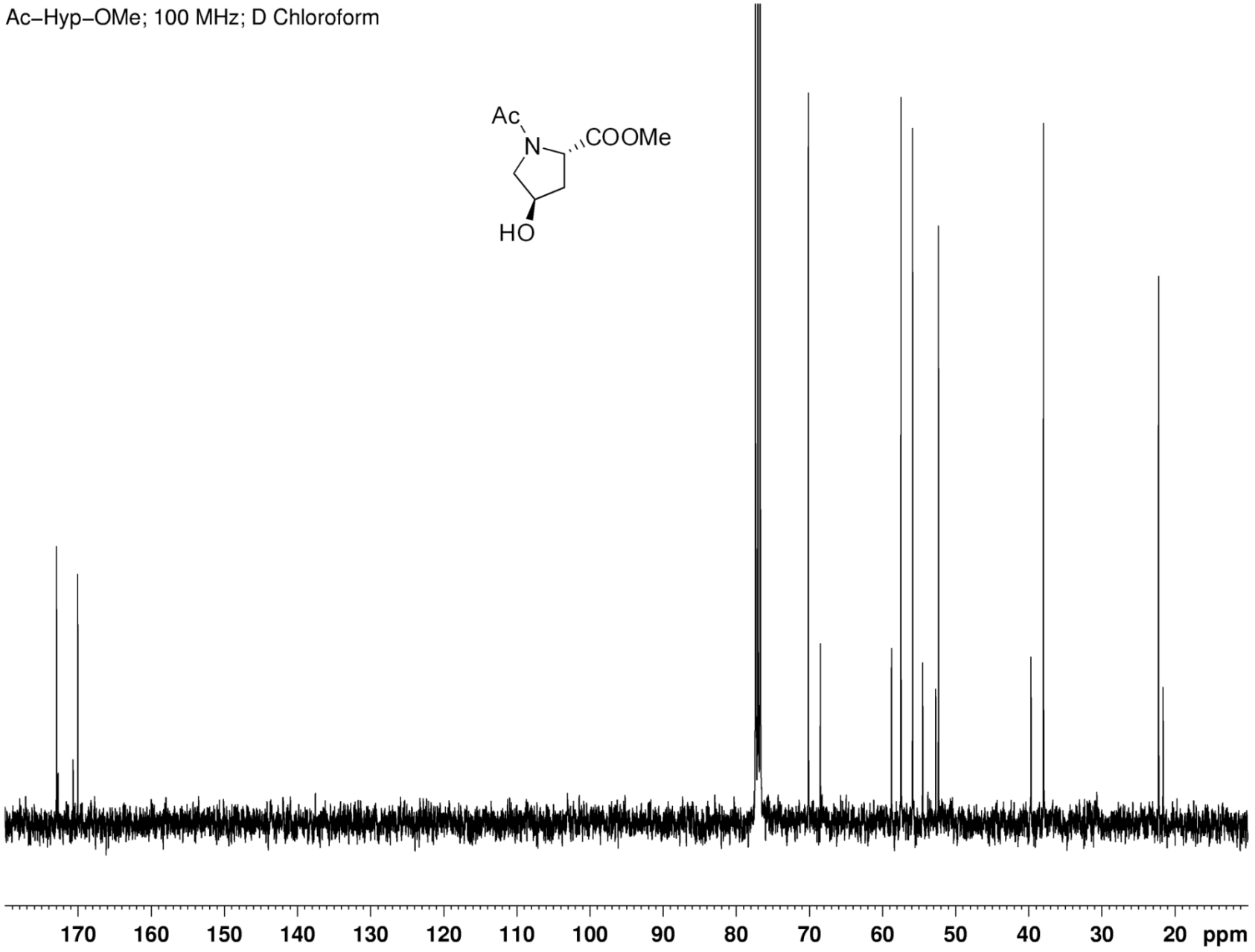
Tetramer (Boc–OAll); 400 MHz; D Chloroform



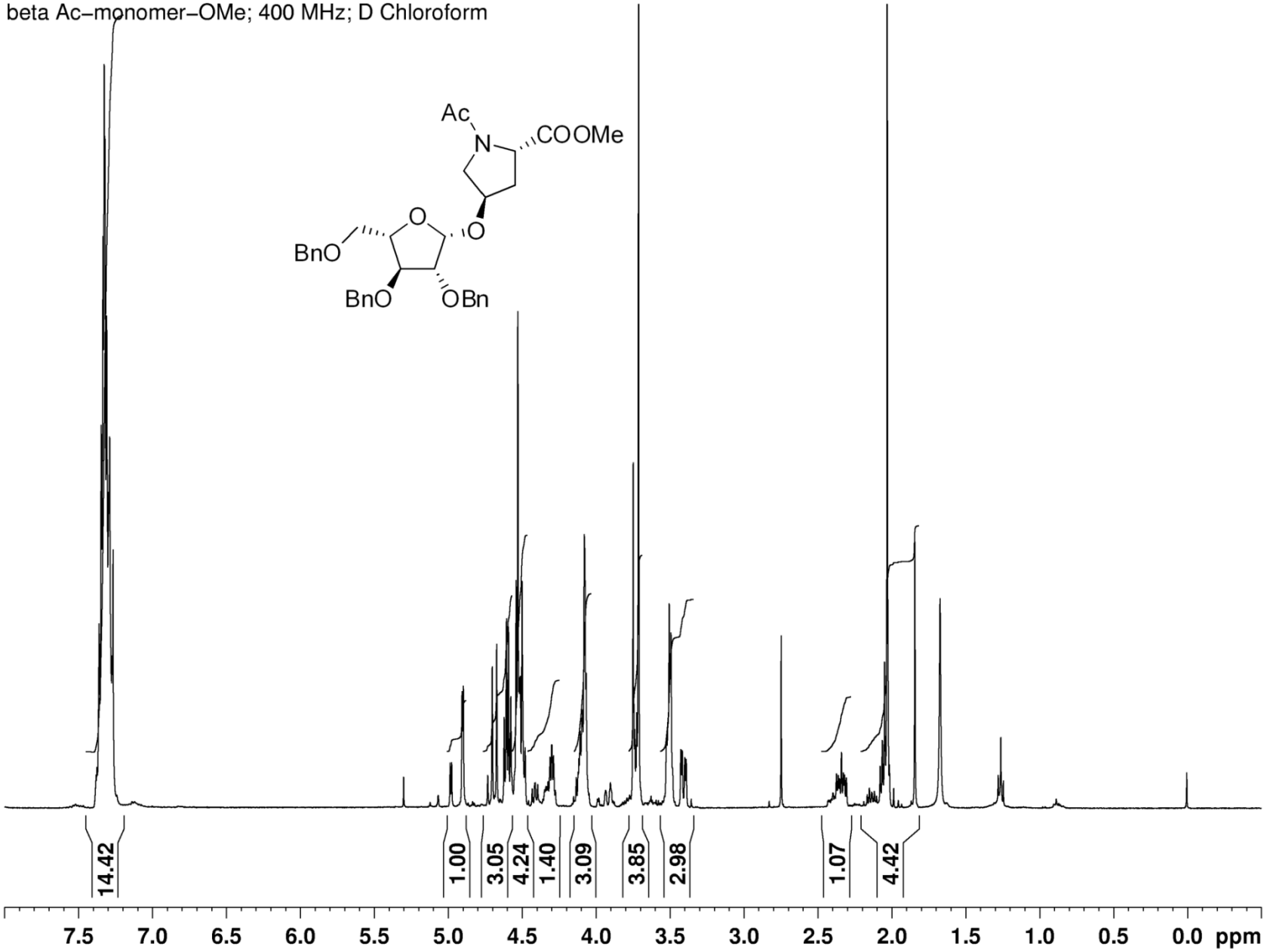
Compound **105** (Scheme 3.24) - ^{1}H NMR spectrum



Compound **105** (Scheme 3.24) – ^{13}C NMR spectrum

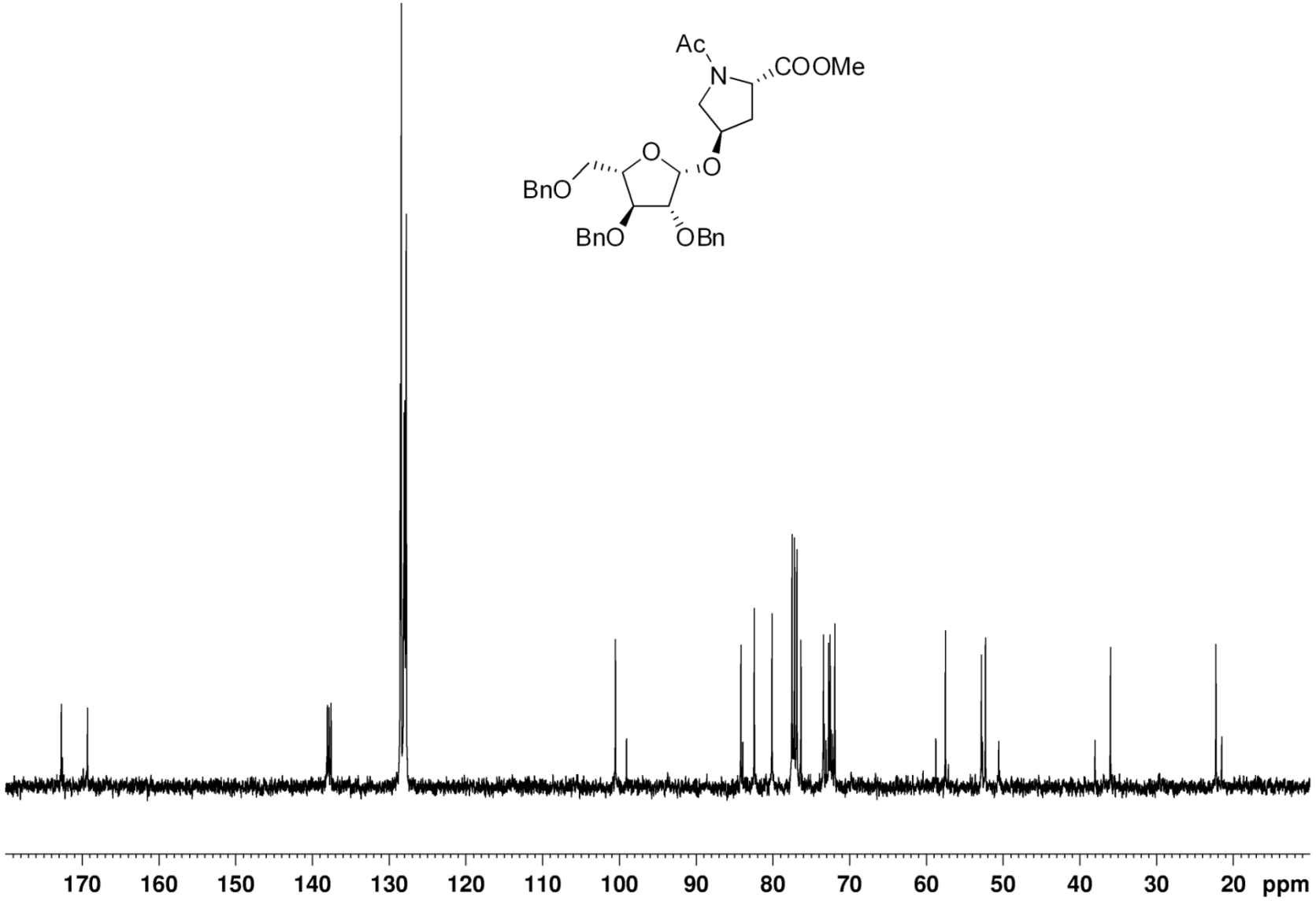


Compound **107** (Scheme 3.25) - ^{1}H NMR spectrum

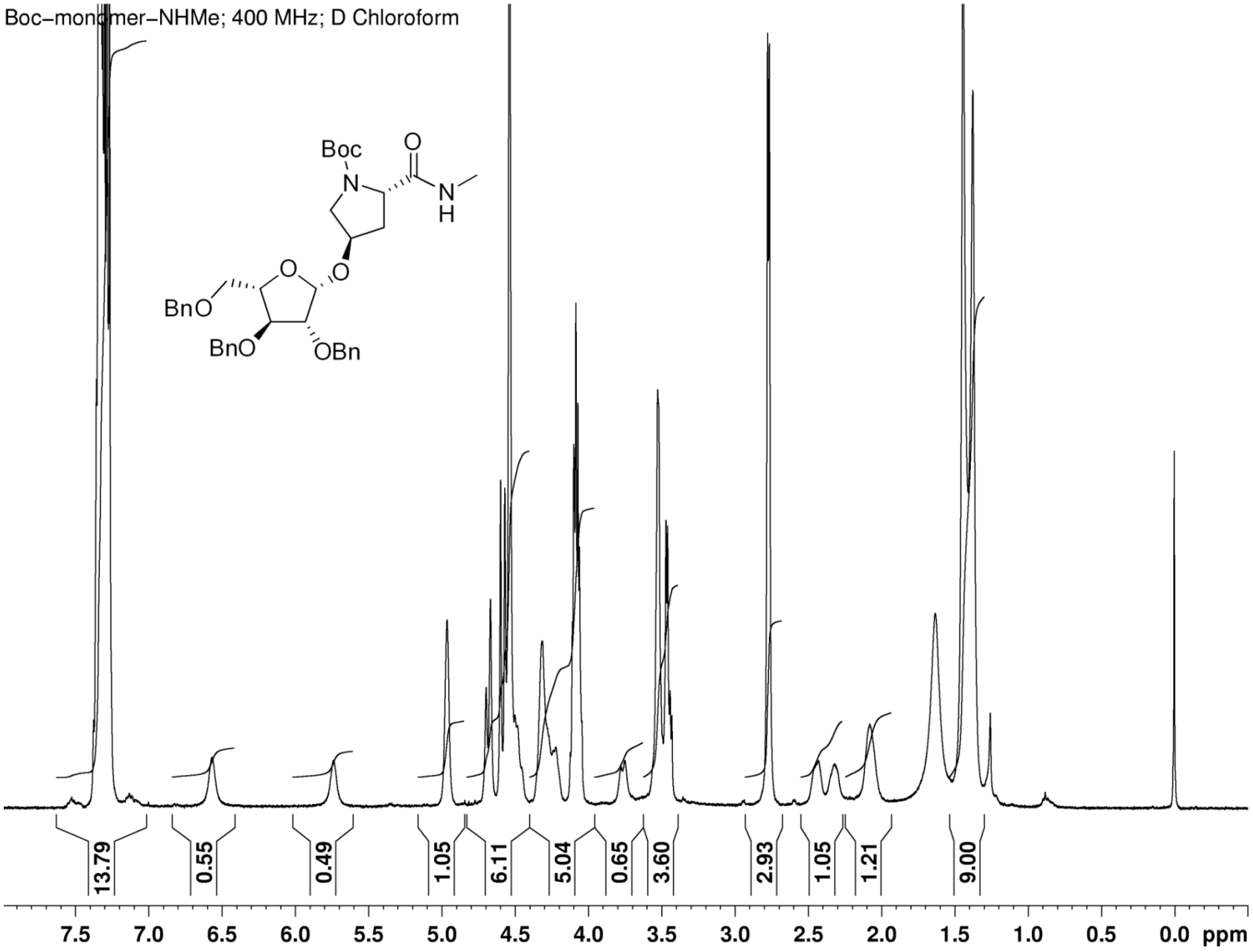


Compound **107** (Scheme 3.25) – ^{13}C NMR spectrum

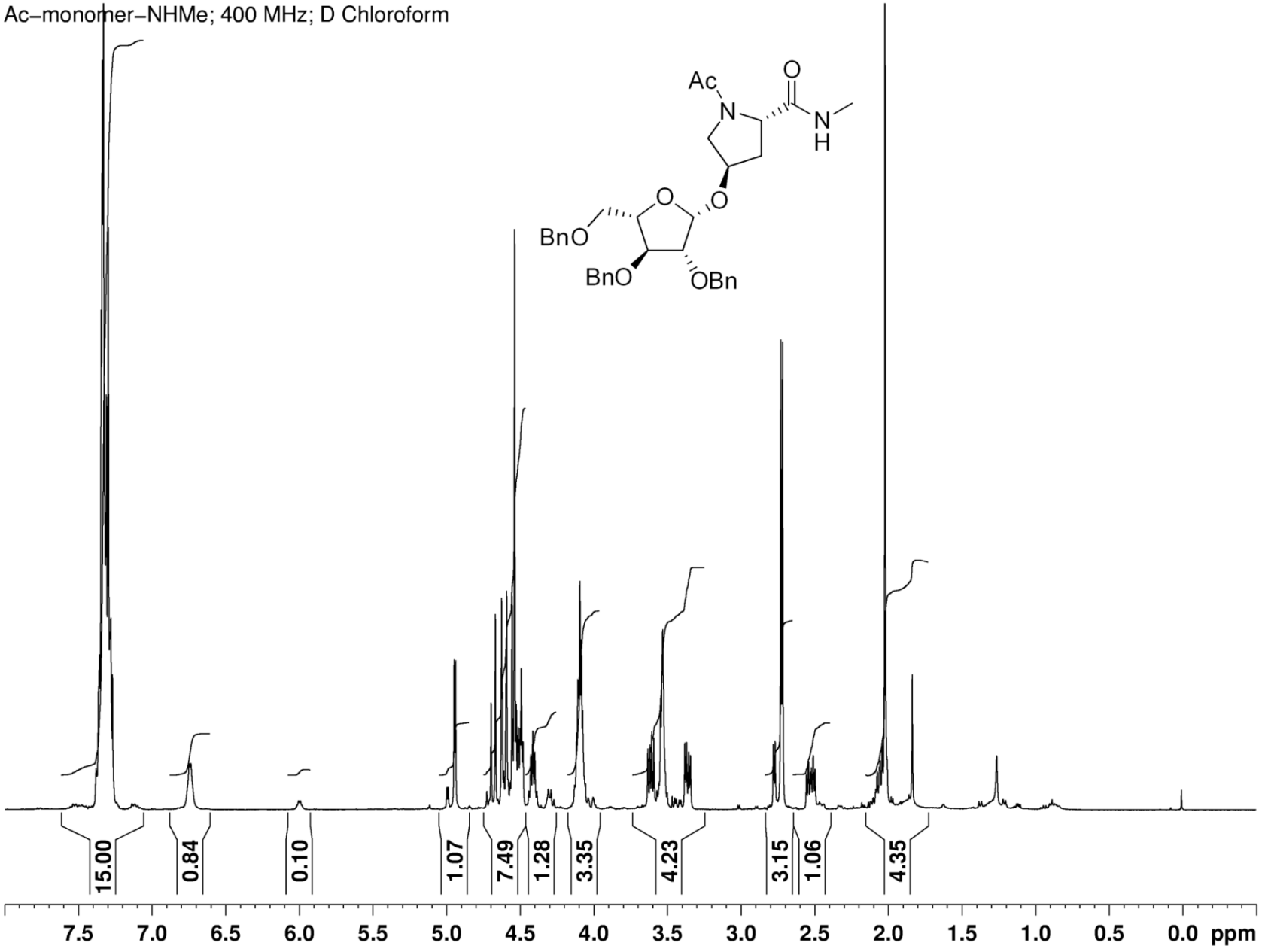
beta Ac–monomer–OMe; 100 MHz; D Chloroform



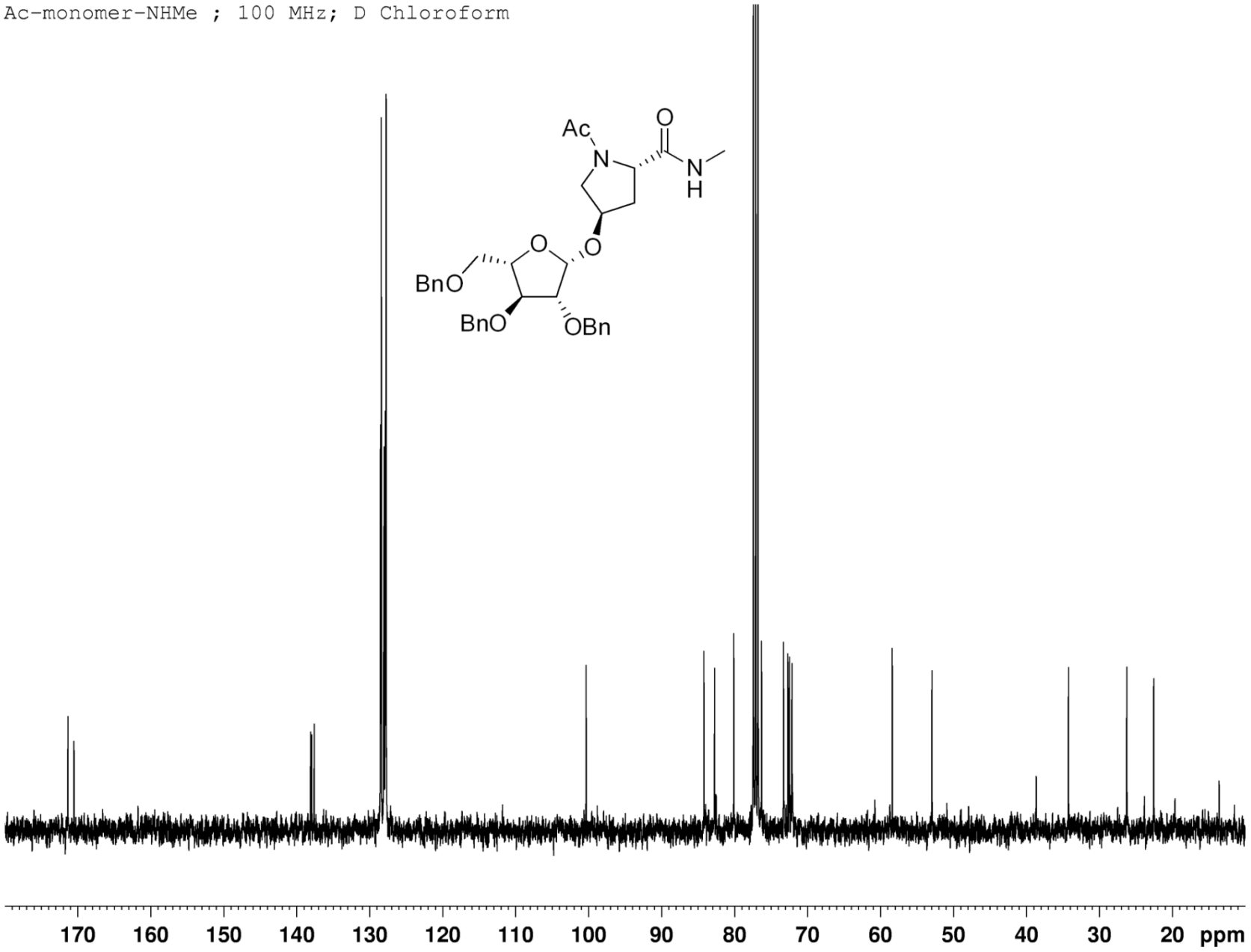
Compound **108** (Scheme 3.25) - ^{1}H NMR spectrum



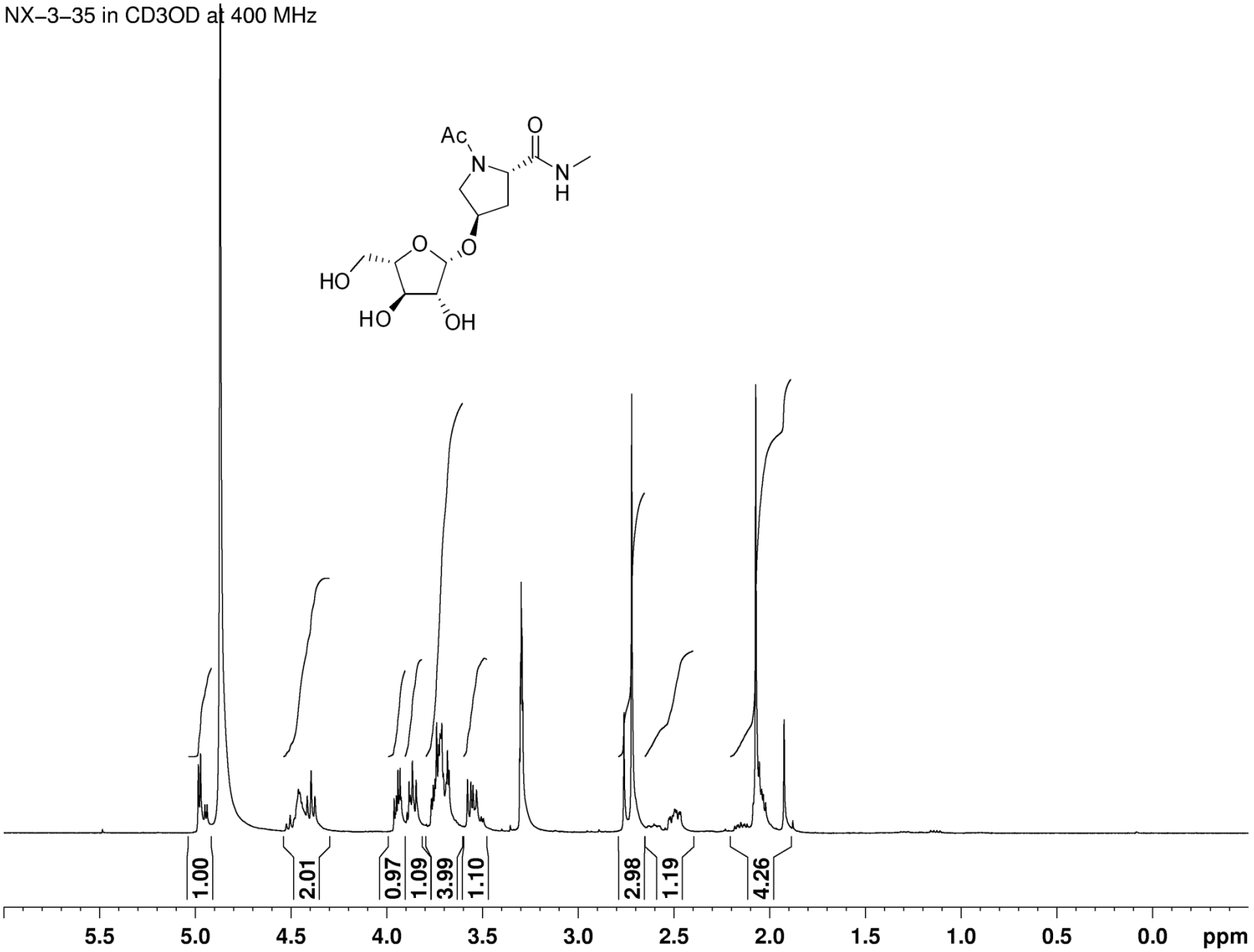
Compound **102** (Scheme 3.21) - ^{1}H NMR spectrum



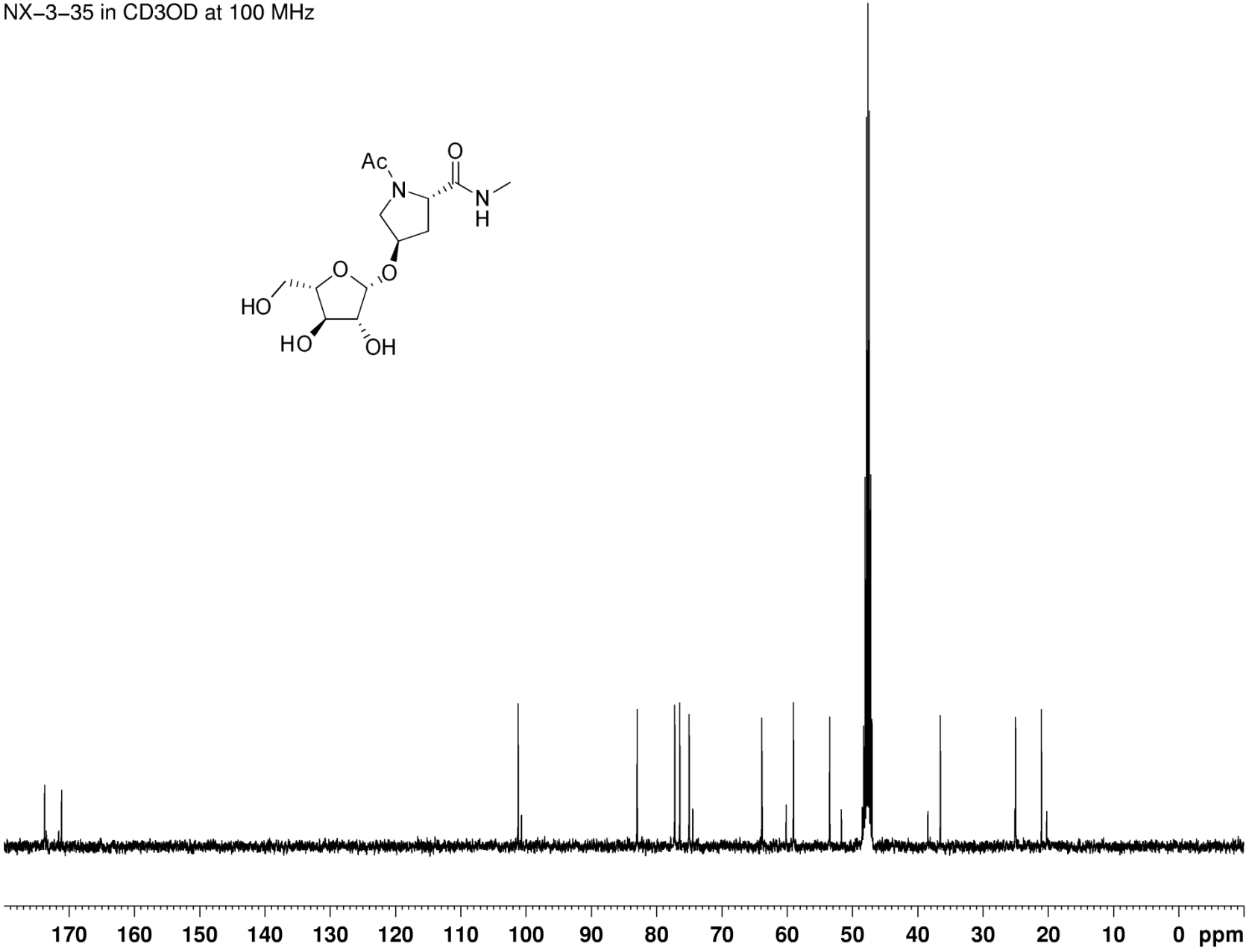
Compound **102** (Scheme 3.21) – ^{13}C NMR spectrum



Compound **118** (Scheme 3.29) - ^{1}H NMR spectrum

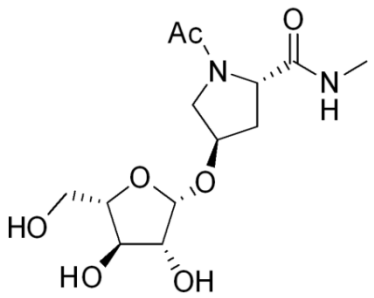


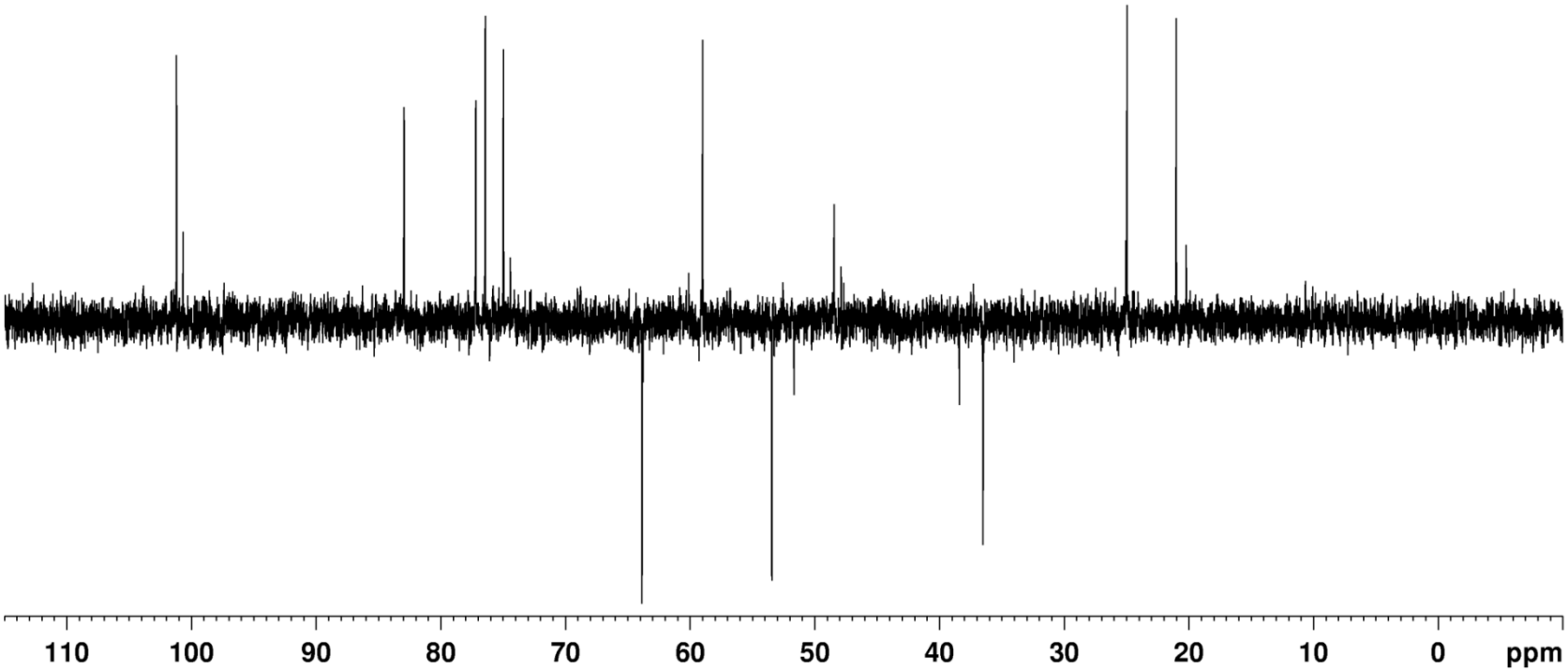
Compound **118** (Scheme 3.29) – ^{1}C NMR spectrum



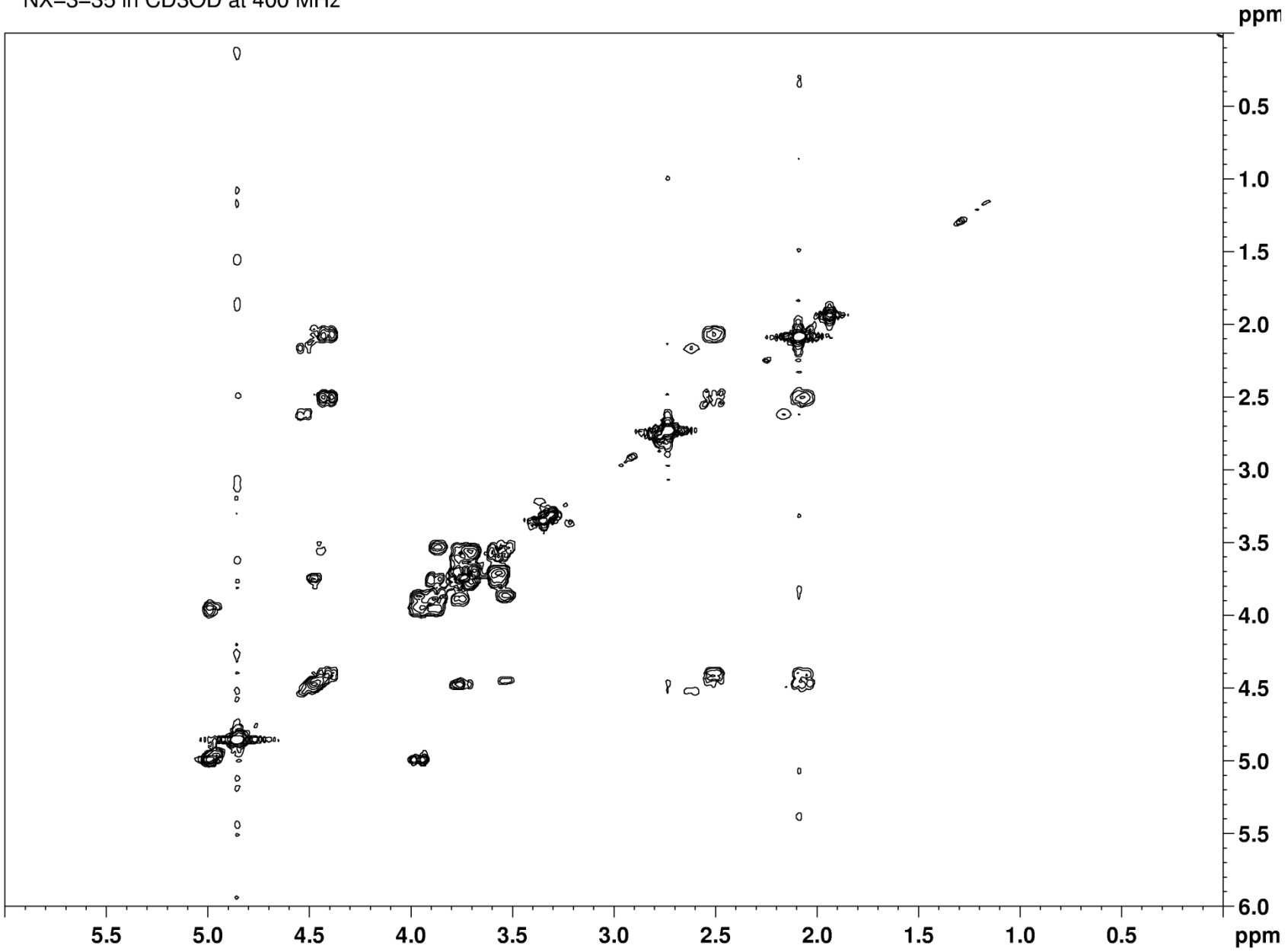
Compound **118** (Scheme 3.29) – Dept 135 NMR spectrum

NX–3–35 in CD3OD at 100 MHz

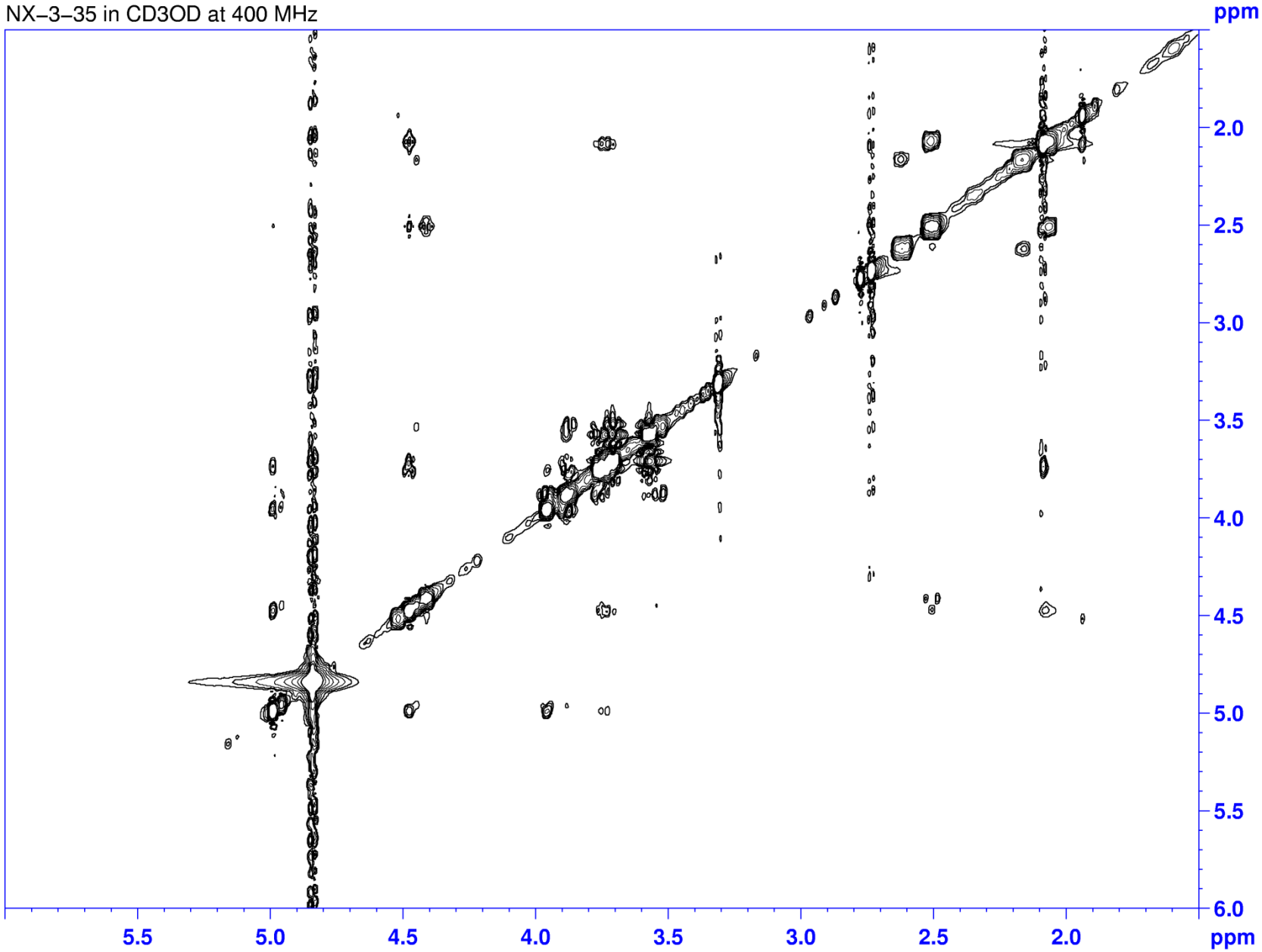




Compound **118** (Scheme 3.29) – COSY NMR spectrum

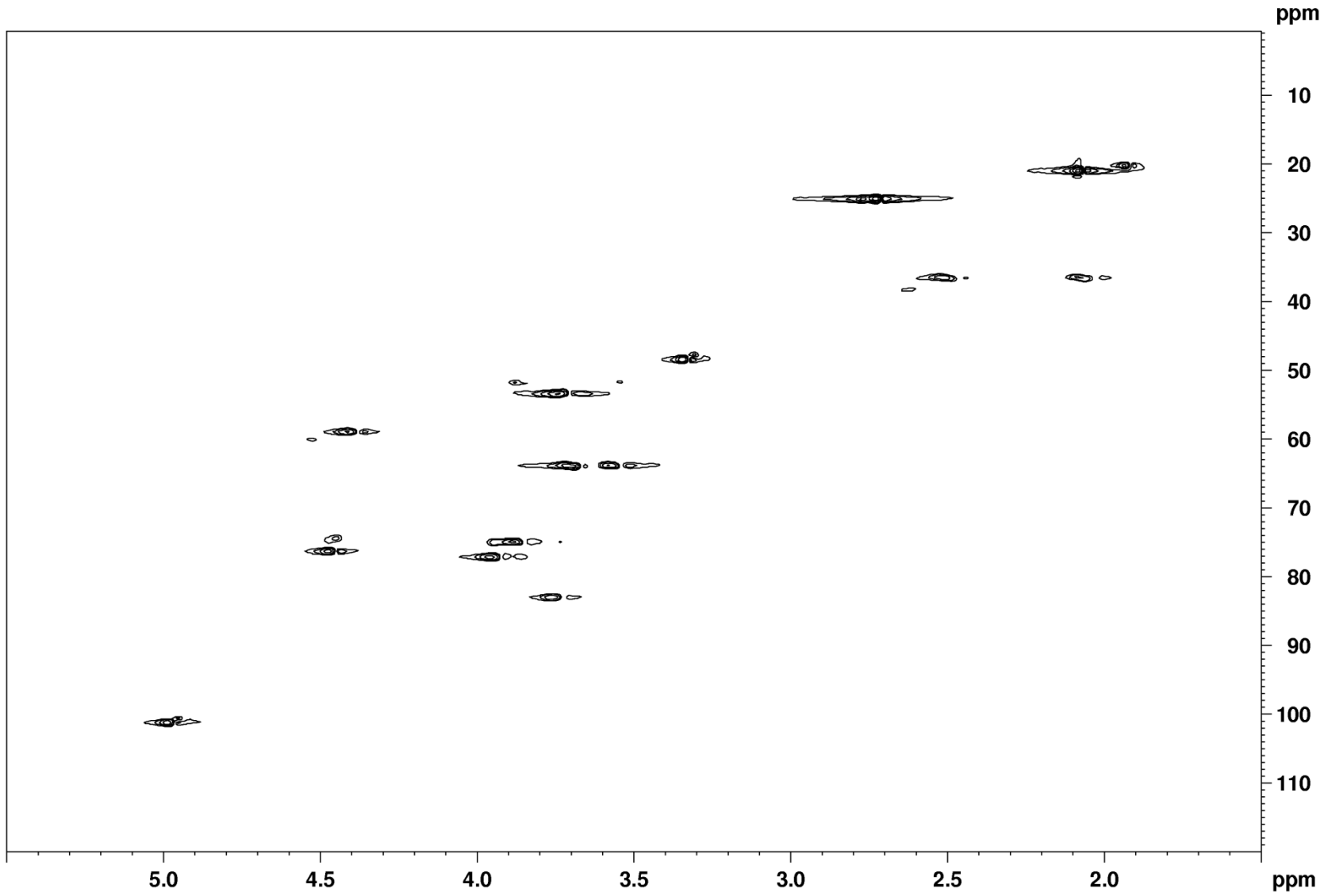


Compound **118** (S cheme 3.29) - NOSEY spectrum

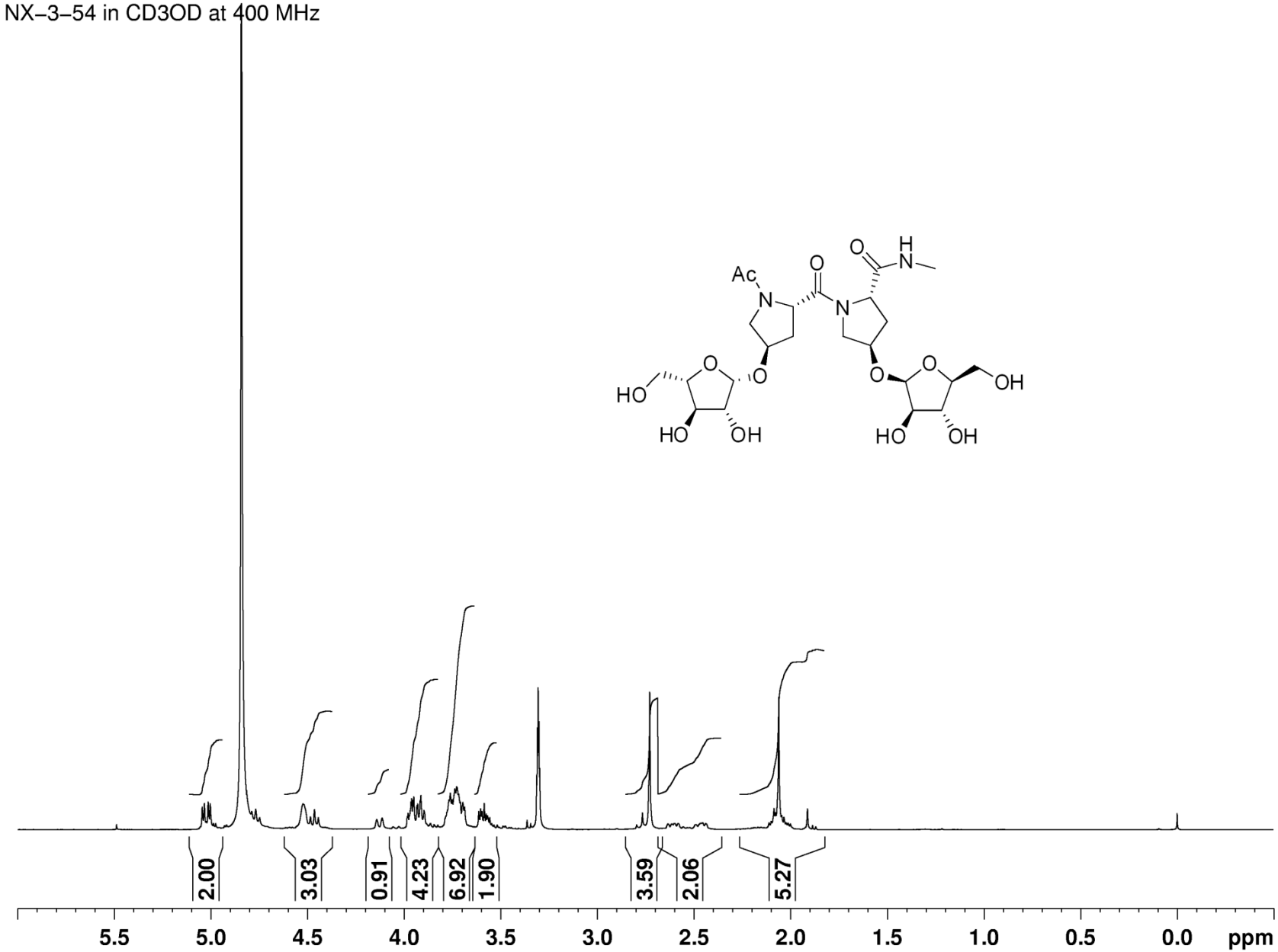


Compound **118** (Scheme 3.29) - HSQC spectrum

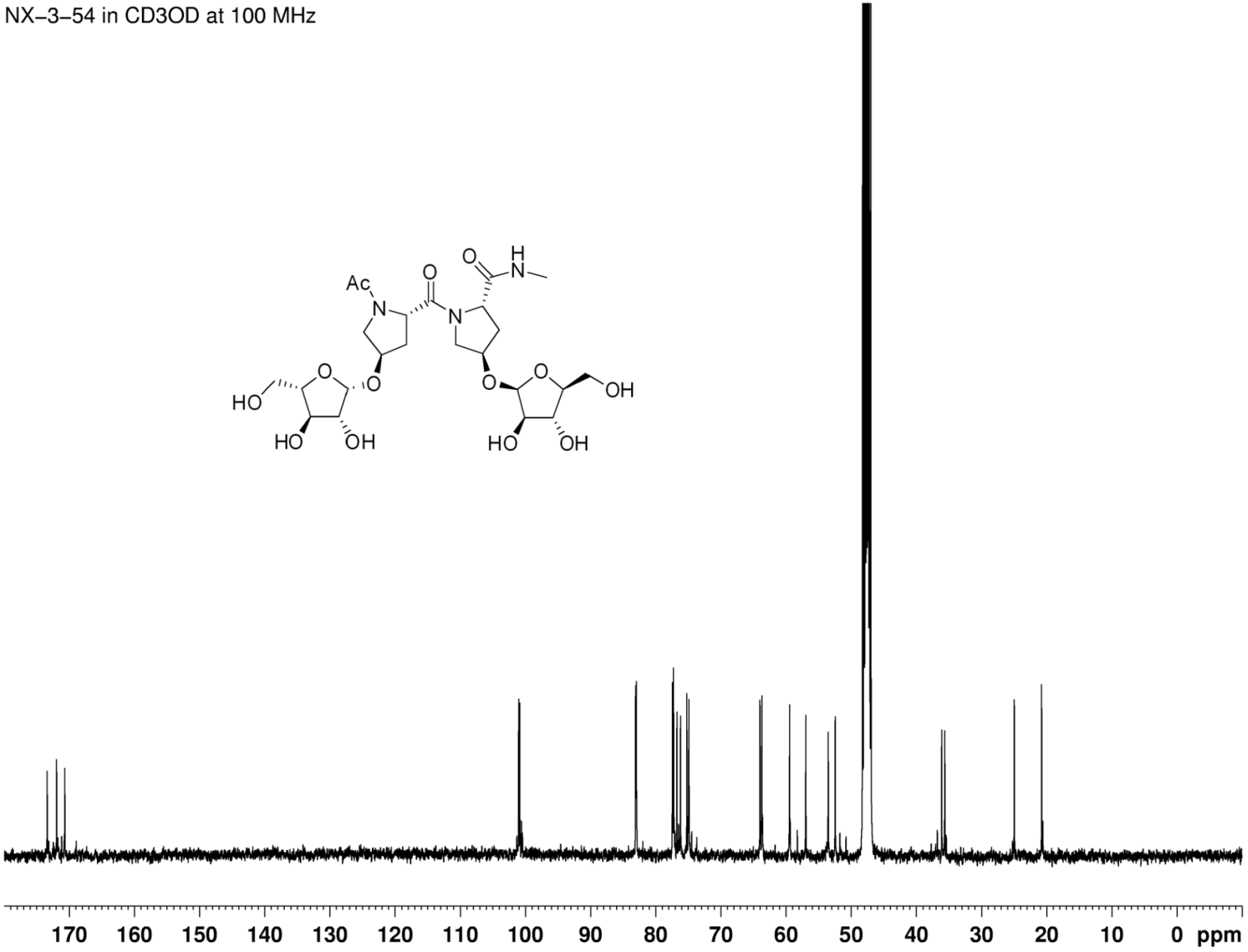
Ac–([beta–L–Araf]Hyp)–NHMe Triol in CD3OD at 400 MHz



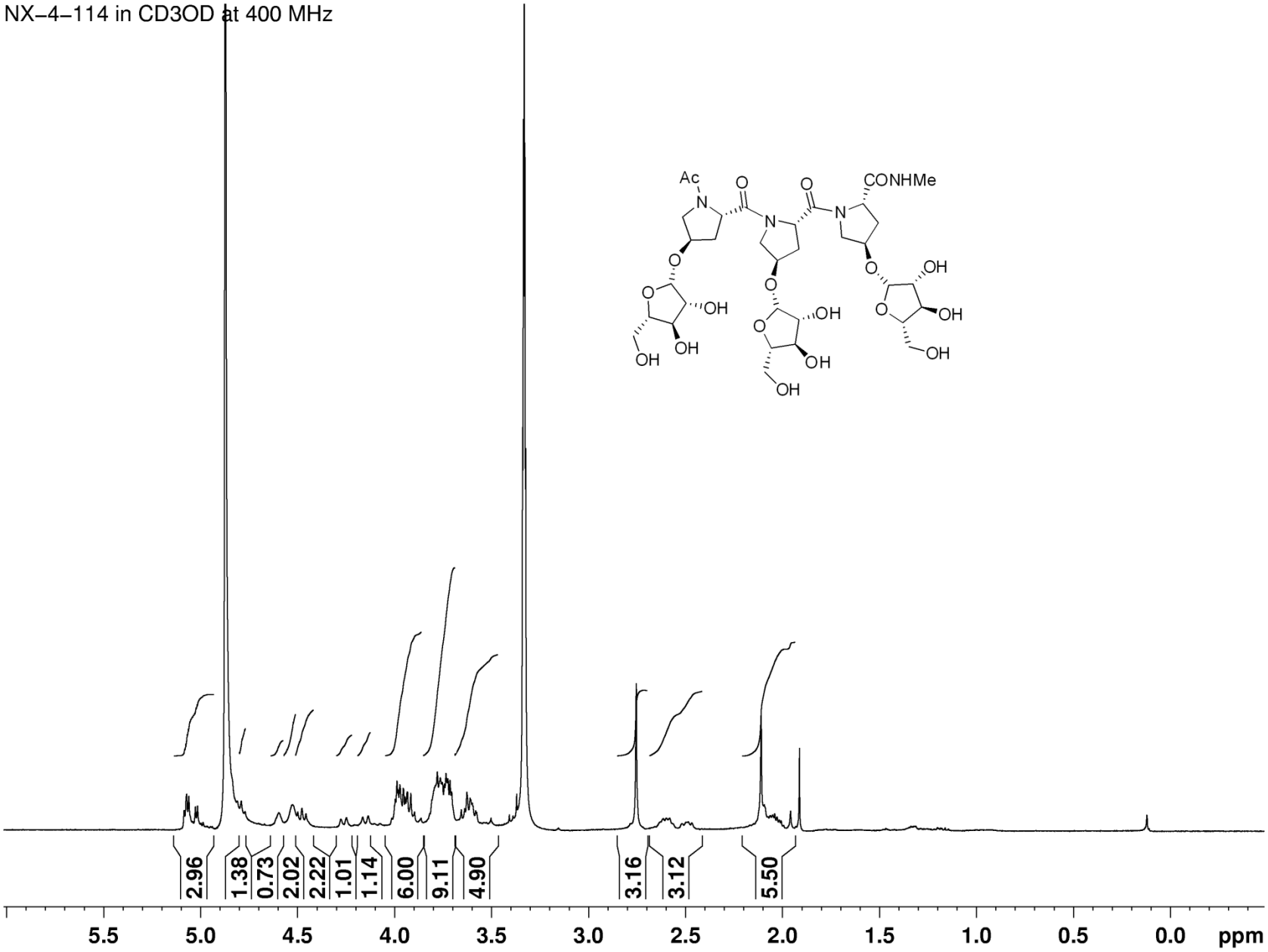
Compound **119** (Scheme 3.29) - ^{1}H NMR spectrum



Compound **119** (Scheme 3.29) – ^{1}C NMR spectrum

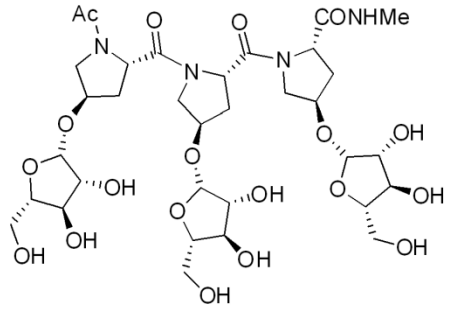


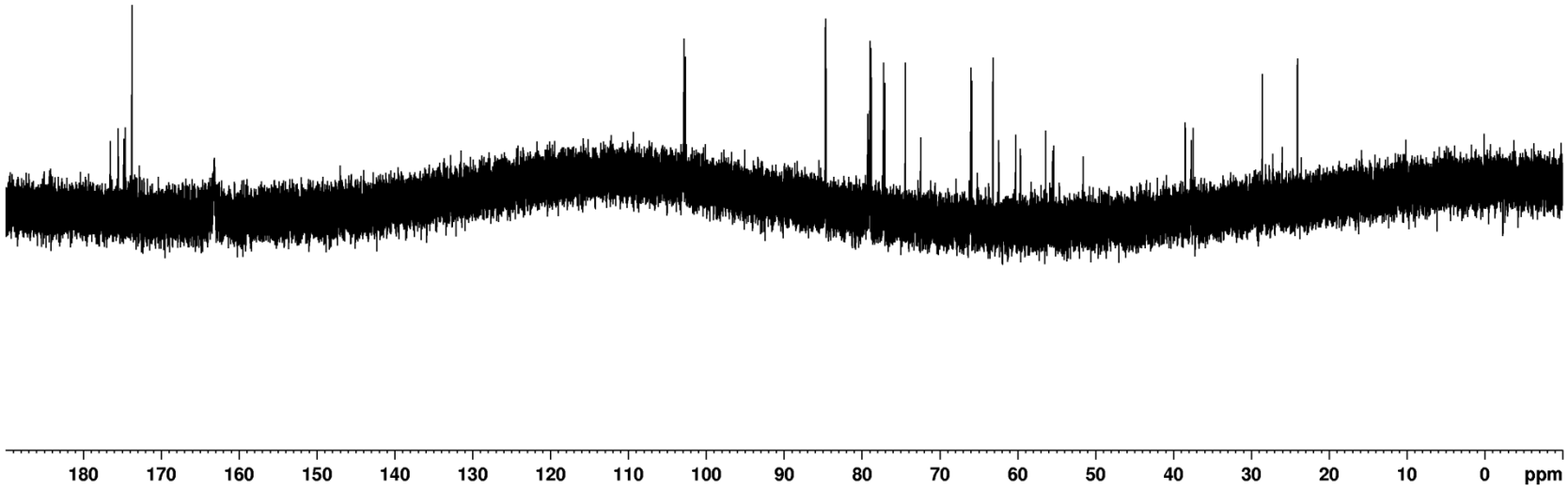
Compound **120** (Scheme 3.29) - ^{1}H NMR spectrum



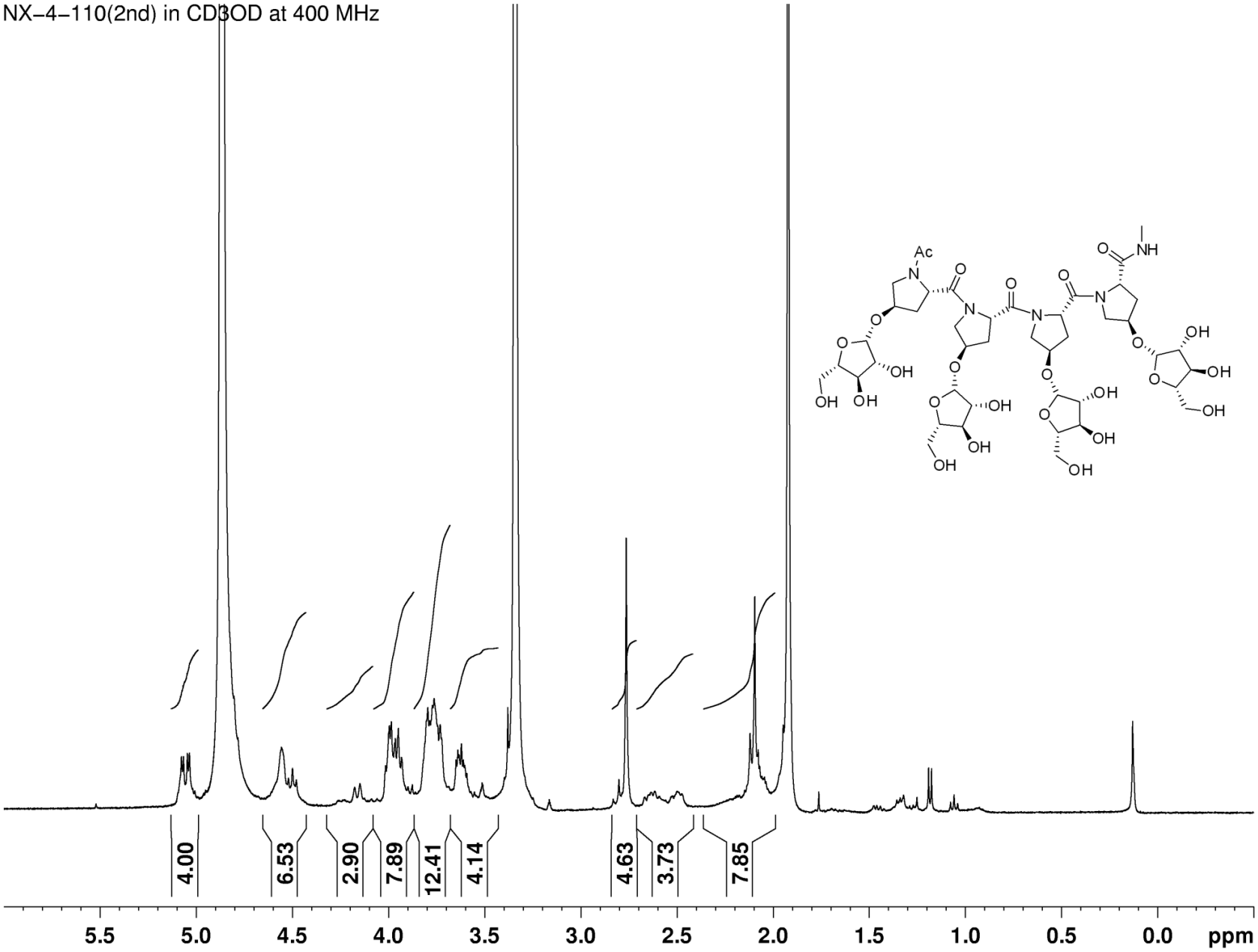
Compound **120** (Scheme 3.29) – ^{13}C NMR spectrum

NX-4-114 in D2O at 175 MHz

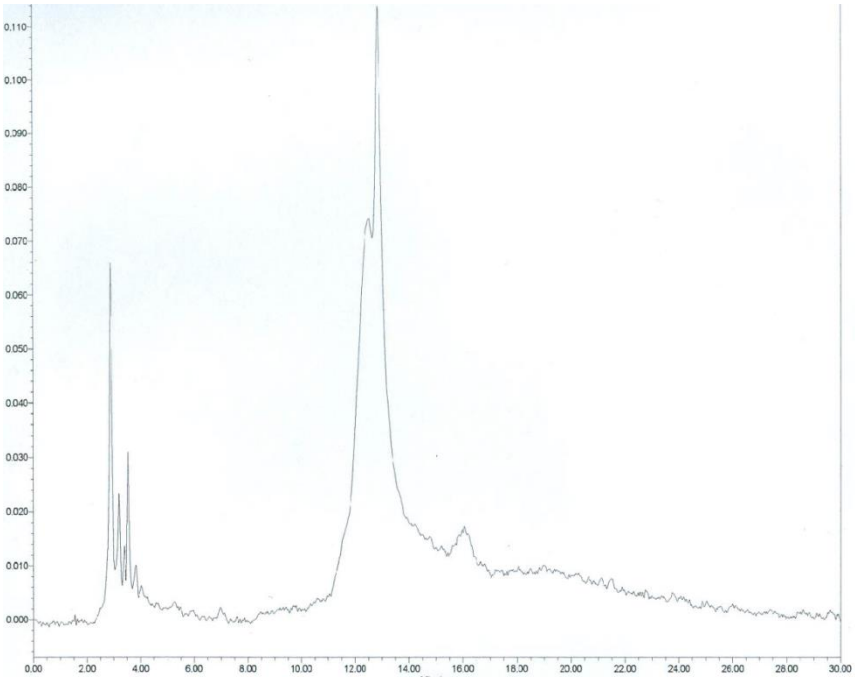




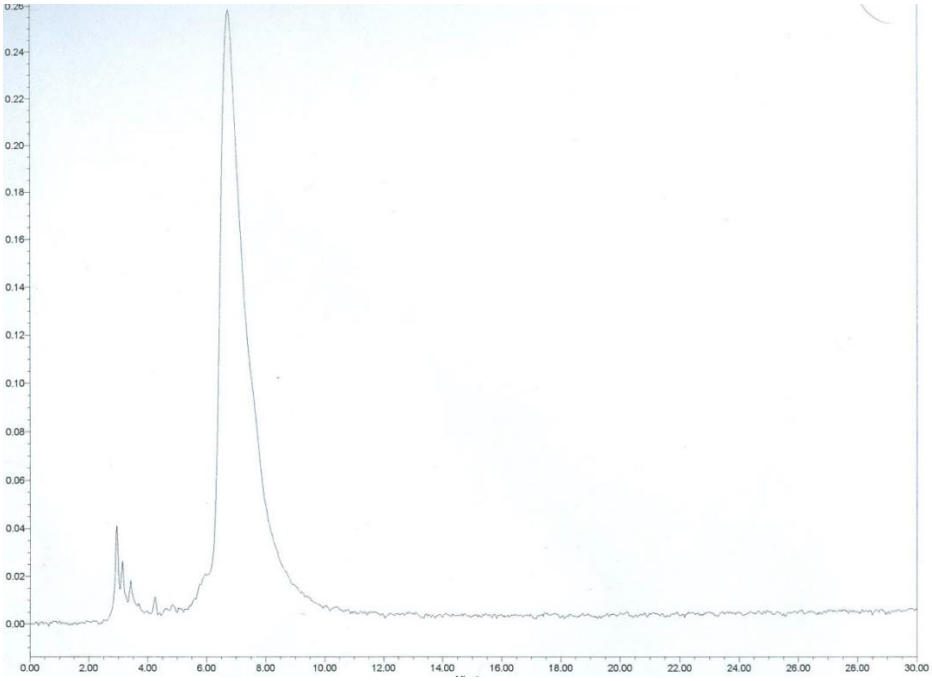
Compound **1** (Scheme 3.29) - ^{1}H NMR spectrum



Triglycotripeptide **99**
1:1 EtOAc/Hex; 5 mL/min; 1 cm silica column



Tetraglycotetrapeptide **100**
2:1 EtOAc/Hex; 5 mL/min; 1 cm silica column



CHAPTER 4: STRUCTURAL STUDIES OF POLYPROLINE GLYCOSIDES

4.1 Circular Dichroism

4.1.1 Polyproline helices

Circular dichroism is a light absorption spectroscopy that gives information about the secondary structure composition of polypeptides and proteins.[88] One such secondary structure found in proteins with recurring proline residues is the polyproline helix (Figure 4.1). A polyproline type I (PPI) helical conformation is a right handed helix having *cis* prolyl amide bonds (ω = 0°). The PPI helix features 3.3 residues per turn with dihedral angles of Φ = -75° and Ψ = +160° (Figure 4.2). A polyproline type II (PPII) helical conformation is a left handed helix featuring all *trans*-amide bonds (ω = 180°) and three amino acid residues per turn.[89] Each residue in the PPII helix has dihedral angles of Φ = -75° and Ψ = +145°.[90]

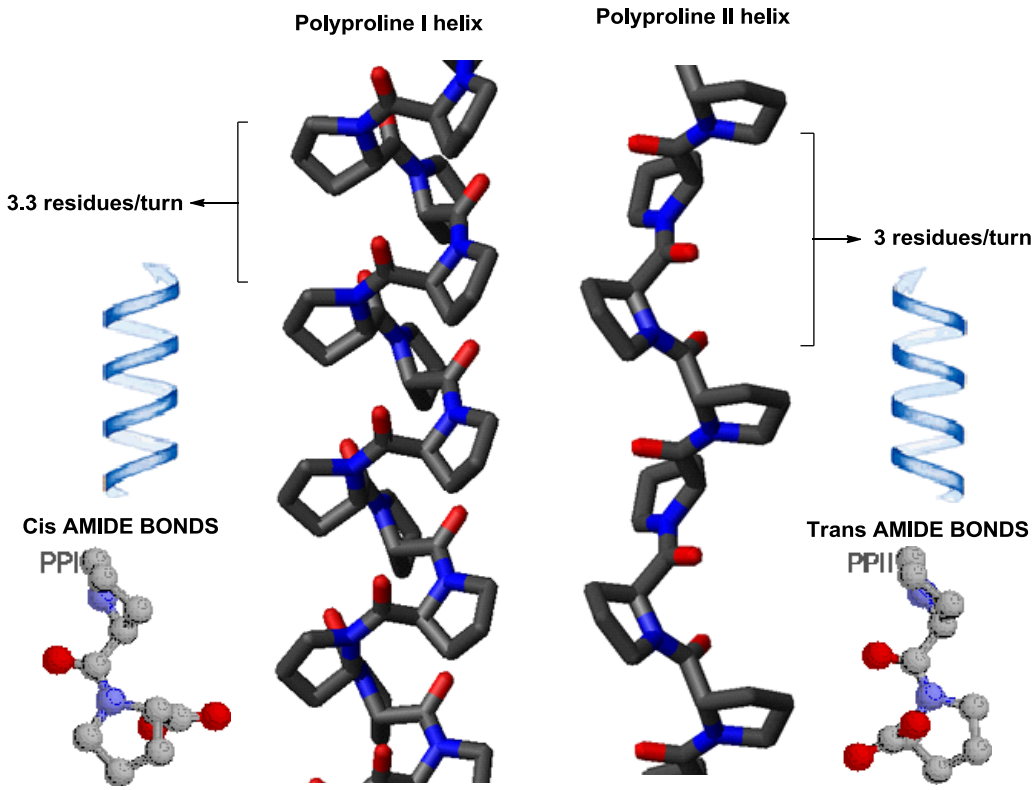


Figure 4.1 Polyproline type I and II helices

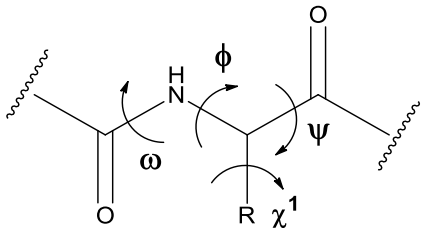


Figure 4.2 Phi (Φ), psi (Ψ), and omega (ω) backbone dihedral angles of peptides

Twenty five percent of all residues in the PPII helix participate in n→π* interactions as reported by Bartlett *et al.*[91] The n→π* interaction arises when a lone pair of electrons on a carbonyl oxygen (n) donates into the empty electrophilic π* orbital on the succeeding carbonyl carbon (Figure 4.3). This electron delocalization event plays a crucial role in the conformational stability of proteins. Proline is especially suited to be an acceptor in this interaction. The psi angle of the PPII conformation is 145°, favorable for n→π* interaction (optimal Ψ = 150°).[92] This n→π* interaction, coupled with the fact that the *trans*-amide bond conformation is lower in energy compared to the *cis*, renders the PPII helix more commonplace than the PPI. Sreerama and Woody estimated that 10% of all proteins may adopt the PPII conformation.[93] Circular dichroism spectroscopy has demonstrated that the PPII conformation has its own distinct elliptical shape (Figure 4.4).[94]

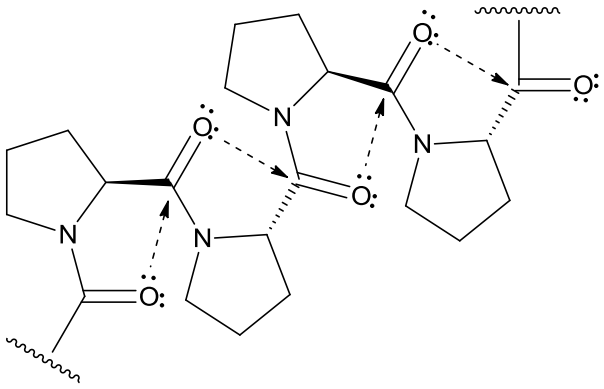


Figure 4.3 n→π* interaction in a proline tetramer.

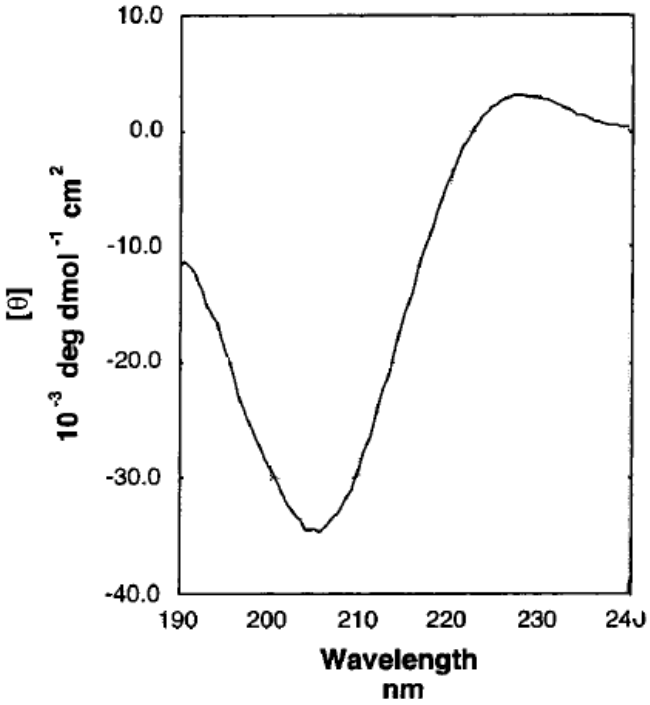
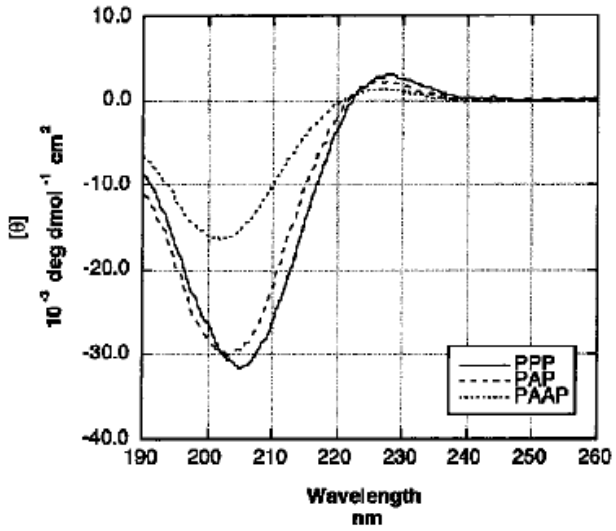


Figure 4.4 CD Spectrum of Ac-$(Pro)_7$-Gly-Tyr-NH_2 taken at 5 °C.[95] Reprint with permission from American Chemical Society.

The PPII structure plays a vital role in numerous biological processes. For example, in the plant kingdom, hydroxyproline-rich glycopeptides (HPRG) are responsible for a plant's growth and defense, among other functions,[96] and are known to adopt a PPII conformation in the cell wall.[97] In the animal kingdom, PPII helices are widely found in collagen, the main component of connective tissues found in vertebrates.[98] Interestingly, through use of computational protein modeling, Himly and coworkers predicted that the carboxyl terminal domain of Art v 1 would exhibit a left handed helical structure resembling collagen.[17] Dedic *et al*. later confirmed that the CD spectra of Art v 1 was indeed similar to those of polyproline helices,[99] although no (further) details of the spectra were provided.

Proline-rich regions of peptides often contain many nonproline residues adjacent to one another.[100] Kelly *et al.* conducted a "host-guest" study on the effect a non-proline residue has on the CD spectra of short polyproline compounds.[95] "Guest" residues were inserted into a host

peptide known to adopt a PPII helix. Alanine was employed due to its inclination to be in a PPII helical conformation. The CD spectra of PPP, PAP, and PAAP were taken at 5 °C (Figure 4.5). The decrease in PPII helical content was found to be three percent between PPP and PAP and nine percent between PAP and PAAP. The nonlinearity of the decreasing helical content suggests that a more pronounced decrease in PPII helical content may be detected in stretches with three or even four non-proline residues in a proline rich region. Despite this decrease, it is apparent from the CD spectra that PAAP still showed significant PPII character.



Ac-Pro-Pro-Pro-Xaa-Pro-Pro-Pro-Gly-Tyr-NH_2

PPP (Xaa = Pro)
PAP (Xaa = Ala)
PAAP (Xaa = Ala_2)

Figure 4.5 Host PPII helix sequence with insertion of guest amino acids. Reprint with permission from American Chemical Society

Figure 4.6 shows the circular dichroism spectra of various H-$(Pro)_n$-OH peptides as reported by Rothe *et al*.[101] Synthetic oligomers of up to 40 unmodified proline residues were investigated. The authors note that three consecutive Pro residues are required for PPII characteristics to be observed. Moreover, the intensity of the maxima, namely the negative maxima at 206 nm, generally increased with the number of Pro residues.

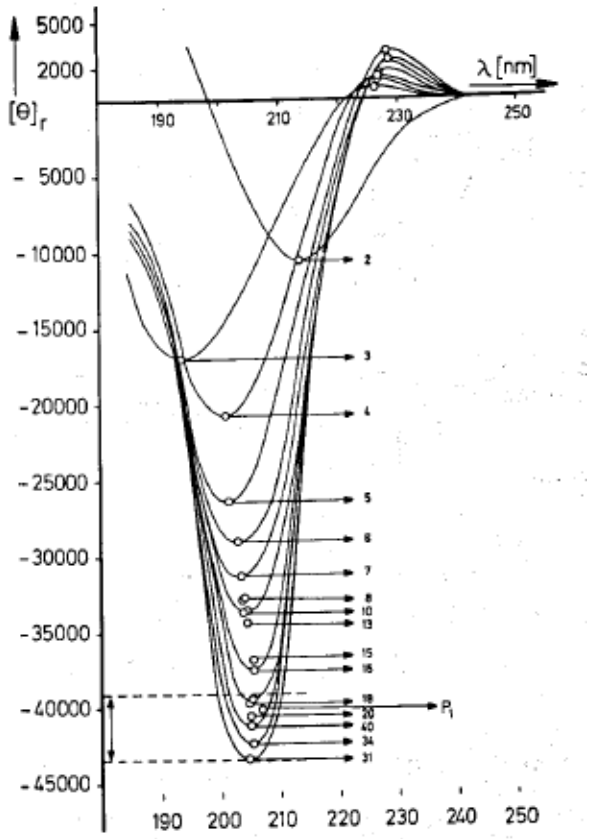


Figure 4.6 CD spectra of synthetic polyproline peptides H-(Pro)$_n$-OH[101]

4.1.2 Effect of pH on peptide conformation

The pH of a solution can be highly influential in the *cis*/*trans* isomerization of X-Pro bonds where there are ionic functional groups.[102] The *cis* conformation allows for an electrostatic interaction between amino and carboxylate groups in the zwitterion (Figure 4.7), while the *trans* conformation is favored in acidic medium due to hydrogen bonding. Thus, the impact of pH on the conformation of peptides having free termini would be measurable for short peptides (≤3 residues) being that the two types of polyproline helices favor differing conformations of proline.[103]

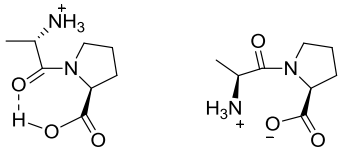


Figure 4.7 *Cis*/*Trans* isomerization of Alanylproline

This effect of pH, however, is not a factor with neutral glycopeptides that are end-capped with terminal amides. We hypothesized that the CD spectra of our monomer and dimer would demonstrate little or no secondary structure. Helbecque *et al.*, in their investigation of H-Gly-$(Pro)_n$-OH peptides, also noted that three proline residues were required for the PPII conformation (n = 3).[103] Thus, the emergence of helical character started with the tetrapeptide (H-Gly-$(Pro)_3$-OH). However, examination of the CD spectrum of their trimer (H-Gly-$(Pro)_2$-OH, Figure 4.8) shows some characteristics of the PPII elliptical curve, although not as intense as the larger oligomers. Hence, we might expect some PPII helical content in our own trimer (Ac-[(β-*L*-Ara*f*)Hyp]$_3$-NHMe) as all of our amino acids involved are prolyl amides and PPII helical formation is a local folding event.[104] Being that the PPII conformation arises from the restricted set of the dihedral angles of an amino acid by the succeeding proline residue,[105] we therefore fully expect the tetramer to exhibit PPII helical character.

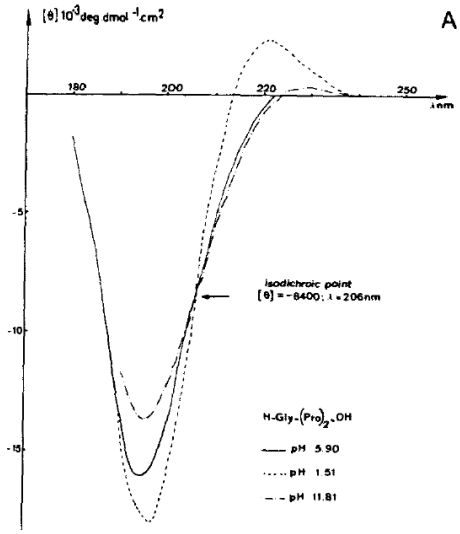


Figure 4.8 CD Spectra of H-Gly-$(Pro)_2$-OH. Reprint with permission from John Wiley and Sons.

4.1.3 Glycosylated oligomers of proline

The Schweizer group published a paper in 2010 detailing the conformation of contiguous β-*O*-galactosylated *trans*-4-hydroxyproline through analysis of far-ultraviolet circular dichroism spectra.[71] As described in Chapter 3, model polyproline peptides were synthesized by solid phase peptide synthesis (Scheme 3.3). Far-ultraviolet circular dichroism spectra of peptides Ac-$(Pro)_9$-NH_2, Ac-$(Hyp)_9$-NH_2, and Ac-$[(\beta\text{-D-Gal})Hyp]_9$-$NH_2$ (**121-123**) were recorded at 25 °C in water (Figure 4.9). All peptides exhibited spectra characteristic of the PPII conformation,[106] *viz.* positive maxima at 220-230 nm and negative maxima at 200-210 nm.[94] The hydroxylated nonamer **122** had a more accentuated positive maxima and a depreciated negative maxima relative to the proline nonamer **121**. The glycosylated nonamer **123** exhibited both a weaker positive and weaker negative band compared to its hydroxyproline counterpart **122.** Owens *et al*. speculated that while the lowered positive and negative maxima of compound **123** may be attributed to a possible distortion of the PPII conformation, much like the PAAP model peptide, glycopeptide **123** was still considered to have significant PPII character.

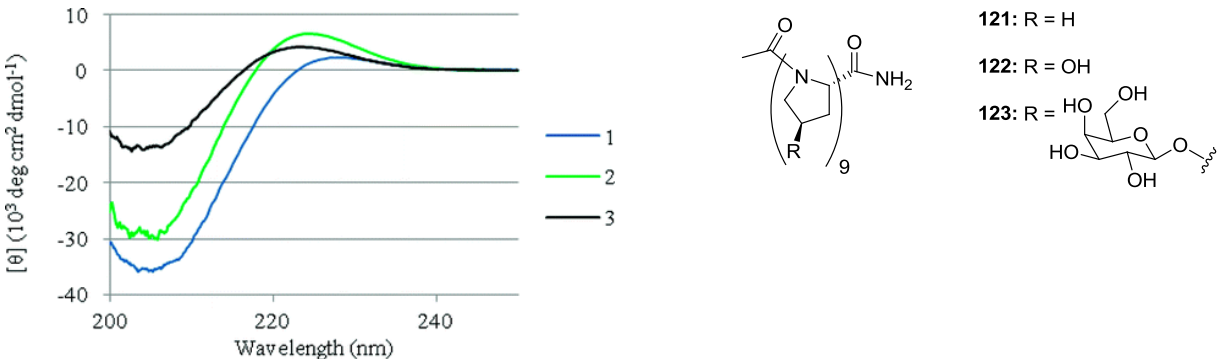


Figure 4.9 CD spectra of model peptides. Reprinted with permission from American Chemical Society.

The relative band strength (ρ) is defined as the ratio of the maximum positive ellipticity to the maximum negative ellipticity.[107] Pysh attributed the increase or decrease in ρ to conformational differences or changes to solvent and carbonyl backbone interactions.[106]

Calculations show that a decreasing ρ value corresponds to an increasing solvent-carbonyl interaction. Owens reported the ρ values of Ac-$(Pro)_9$-NH_2, Ac-$(Hyp)_9$-NH_2, and Ac-[(β-D-Gal)*Hyp*]$_9$-NH_2 to be 0.06, 0.21, and 0.29 respectively.[71] The consistent increase in ρ through the series of model peptides can be attributed to a decreasing solvation of the amide backbone. While the increase in ρ values can also indicate a destabilization of the PPII conformation, the authors hypothesized that hydration differences, or shielding of the amide carbonyl groups, was the primary reason for the significant differences in ρ values.

Naziga *et al.*, working in conjunction with the Schweizer group, recently reported that the conformation of glycosylated oligoprolines is highly influenced by solvent interactions.[108] Examining the same model peptides as Owens *et al.* (Figure 4.9, **121**, **122**, and **123**),[71] Naziga and coworkers used molecular modeling techniques to investigate the increase in thermal stability of glycosylated oligoprolines and the effect of temperature on the polyproline conformation. Thermal melting experiments gave T_m values of 22, 38, and 70 °C for compounds **121**, **122**, and **123** respectively.[71] While increased stability of the Hyp and glycosylated Hyp oligomers relative to the unmodified proline oligomer can be attributed to a higher population of the *trans* isomer,[109] it did not explain the T_m difference in Ac-$(Hyp)_9$-NH_2 and Ac-[(β-D-Gal)Hyp]$_9$-NH_2 as *trans* stabilization was not observed upon glycosylation of Ac-Hyp-NHMe as a model compound. Using molecular dynamics simulations, Naziga *et al.* reported that while sugar-sugar and sugar-backbone interactions may contribute to the stability of the PPII conformation, it was the interaction between the sugar and water molecules that was paramount to the increased stability of the glycosylated oligoprolines.[108] The free energy difference between the Hyp and glycosylated Hyp oligomers was found to be 26 kcal mol^{-1} in explicit solvent (*c.f.*, no estimated difference in implicit solvent where hydrogen bonding is not accounted for).

4.1.4 CD Spectra of Ara-Hyp glycopeptides

Circular dichroism spectra of the monomer **118**, dimer **119**, trimer **120**, and tetramer (**1**) were taken in the far-ultraviolet region of the spectrum (190-240 nm). The aqueous solutions of monomer, dimer, trimer, and tetramer showed a pH reading of 6.80, 7.38, 8.79, and 9.48 respectively. Circular dichroism data was recorded in water at 20 °C at a concentration of ~0.4 mM.

Examination of the CD spectrum of our monomer showed that this compound is largely unordered, as we had expected (Figure 4.10). While there seems to be a negative maxima at 200 nm ([θ] = -3967 deg cm^2 $dmol^{-1}$), there is no defined maxima. A PPII helix would feature a positive maxima around 220-230 nm, whereas the spectrum of the monomer never rises above zero deg cm^2 $dmol^{-1}$. The CD spectrum of the monomer is akin to that of an unmodified proline dimer (Figure 4.9, n = 2) where H-$(Pro)_2$-OH exhibits a negative maxima at around 210 nm but no positive maxima.[101] This is consistent with previous work reporting that peptides with less than three residues do not exhibit the PPII conformation.[101]

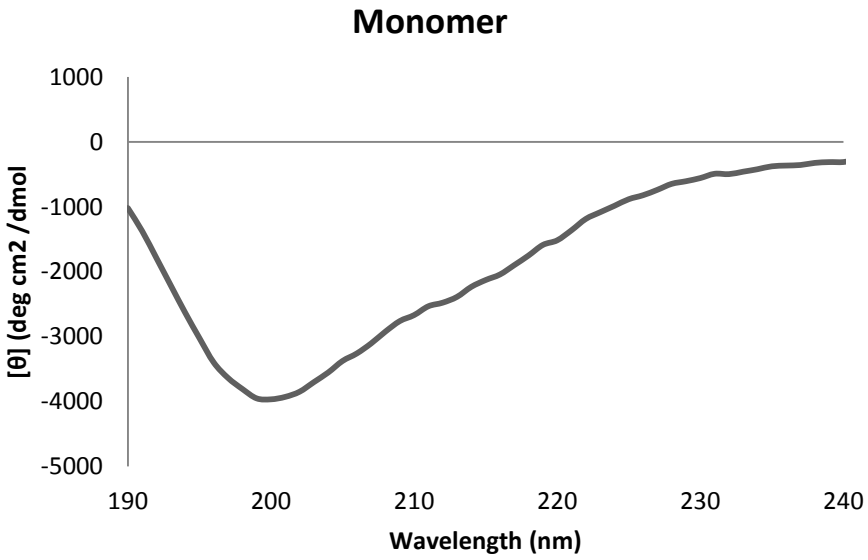


Figure 4.10 CD spectrum of Ac-([β-L-Ara*f*]Hyp)-NHMe

We fully expected that the CD spectrum of the dimer would be similar to that of the unordered monomer. Surprisingly, a cursory glance at Figure 4.11 shows that the dimer does indeed exhibit order in its elliptical curve. The peptide Ac-([β-L-Ara*f*]Hyp)$_2$-NHMe displayed both a positive band (λ_{max} = 220 nm, [θ] = 2905 deg cm^2 dmol^{-1}) and a negative band (λ_{min} = 199 nm, [θ] = -10423 deg cm^2 dmol^{-1}) that is characteristic of the PPII conformation.[94] This is significant as previous studies on short polyproline peptides have shown that formation of the PPII helix began at three proline residues (H-Pro$_3$-OH) (Figure 4.9, n = 3).[101] It is tempting to suggest that this difference is due to the carbohydrate residues acting as a restrictive medium, thus giving more order to the prolyl component of the molecule. Considering that polyproline helices are stabilized by the steric constraint of their pyrrolidine rings, we believe that the added bulk of the sugars might very well be contributing to their heightened structure.

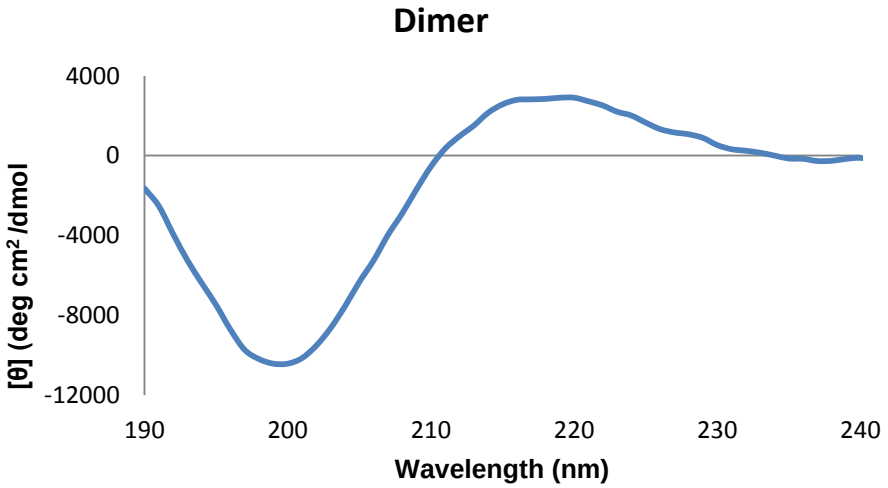


Figure 4.11 CD spectrum of Ac-([β-L-Ara*f*]Hyp)$_2$-NHMe

The CD spectra of both trimer and tetramer showed typical PPII-type helical structure (Figure 4.12). Peptide Ac-([β-L-Ara*f*]Hyp)$_3$-NHMe showed a positive band at 222 nm ([θ] = 4207 deg cm^2 dmol^{-1}) and a negative maxima at 203 nm ([θ] = -13352 deg cm^2 dmol^{-1}). Peptide Ac-

([β-L-Ara*f*]Hyp)$_4$-NHMe exhibited a positive band at 220 nm ([θ] = 3704 deg cm^2 dmol^{-1}) and a negative maxima at 200 nm ([θ] = -13816 deg cm^2 dmol^{-1}).

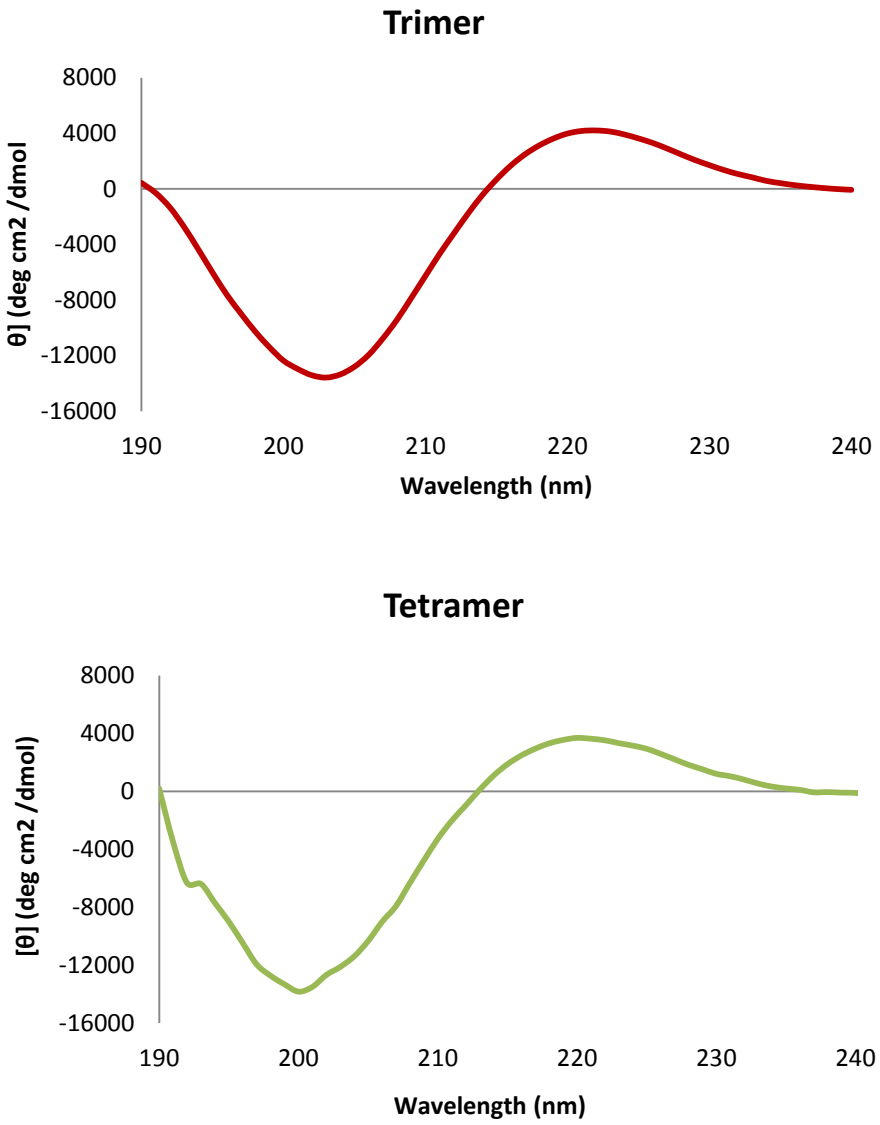


Figure 4.12 CD spectrum of Ac-([β-L-Ara*f*]Hyp)$_3$-NHMe and Ac-([β-L-Ara*f*]Hyp)$_4$-NHMe

Figure 4.13 shows an overlay of the CD spectra of all four synthetic glycopeptides relative to one another. Using the monomer as a baseline value, we can see that the intensity of the negative maxima increases with the length of the glycopeptide. This is consistent with the findings of Rothe.[101] The trimer and tetramer, as expected, showed the most intense positive

and negative bands. This is likely due to the fact that the larger oligomers possess the necessary number of residues to form a proper PPII helix.

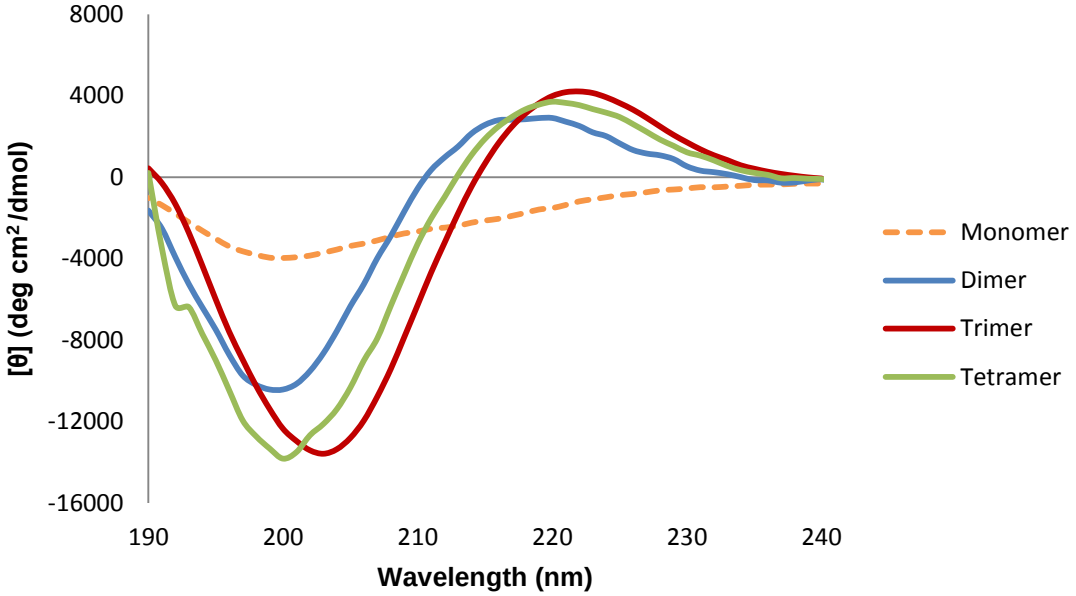


Figure 4.13 CD Spectra of all four synthetic glycopeptides

The relative band strength (ρ value) of dimer, trimer, and tetramer are 0.28, 0.30, and 0.27 respectively. While we anticipated a decrease in relative band strength with increasing residue count, this was not the case. Instead, the ρ value increased slightly from dimer to trimer, with tetramer having the lowest ρ value of all three peptides. However, we do not believe the differences in these values are significant. The discrepancies in the ρ values are most likely due to common experimental error and it is probable that the PPII helical content of the three compounds are quite comparable to one another. These values are also in agreement with the ρ value of the galactosylated hydroxyproline nonamer reported by Owens (ρ = 0.29).[71]

4.2 Nuclear Magnetic Resonance Spectroscopy

4.2.1 Characterization of Ara-Hyp glycopeptides of nArt v 1 by Leonard *et al.*

Nuclear magnetic resonance spectroscopy was conducted on α-arabinosidase-treated nArt v 1. Tables 4.1 and 4.2 show the chemical shifts for Hyp and β-Ara residues as reported by Leonard and coworkers.[22] Three types of β-arabinoside residues were found for which distinct anomeric proton signals could be discerned around 5.10-5.12 ppm (Table 4.2). This is consistent with the occurrence of three residues per turn in the PPII helix. The ^{13}C NMR signal for the anomeric carbon, which could not be differentiated for the three types, was found at 100.9 ppm. For comparison, the C1 signal for α-Ara residues of nArt v 1 were found at 107-109 ppm. Typically, when substitutents on C1 and C2 are *cis* to one another, as is the case for β-arabinosides, the C1 resonances can be found between 100 to 105 ppm.[110] The ^{13}C chemical shifts are also in agreement with literature values reported from naturally occurring polysaccharide arabinans containing the β-Ara motif.[111] Nuclear Overhauser effect (nOe) correlations between the anomeric proton of β-Ara and the γ-proton of Hyp support the linkage of the arabinoside. Leonard *et al.* had reported the first ^{1}H and ^{13}C characterization of contiguous mono-β-arabinosides of hydroxyproline found in the carbohydrate region of nArt v 1.

Table 4.1 NMR resonances of Hyp in Art v 1 [22]

Hyp	**Chemical Shift (ppm)**					
	α	**β1**	**β2**	**γ**	**δ1**	**δ2**
Type 1 ^{1}H	4.837	2.547	2.000	4.581	4.035	3.729
Type 2 ^{1}H	4.844	2.552	2.005	4.574	4.012	3.758
Type 3 ^{1}H	4.831	2.531	2.001	4.580	4.028	3.722
^{13}C	58.6	35.7		77.5	53.8	

Table 4.2 NMR resonances of β-Ara in Art v 1[22]

β-Ara	Chemical Shift (ppm)					
	1	**2**	**3**	**4**	**5a**	**5b**
Type 1 ^{1}H	5.120	4.10	4.012	3.848	3.744	3.600
Type 2 ^{1}H	5.108	4.069	3.991	3.840	3.734	3.577
Type 3 ^{1}H	5.105	4.089	3.972	3.840	3.734	3.572
^{13}C	100.9	77.2	75.3	82.8	64.1	

4.2.2 Characterization of synthetic Ara-Hyp glycopeptides

Various ^{1}H, ^{13}C, and 2-D NMR spectra of Ac-([β-L-Ara*f*]Hyp)-NHMe (**118**) in CD_3OD were acquired. Due to the rotational isomerization of the prolyl acetamide, an approximate 4:1 ratio of rotamers is detected. Examination of the NOESY spectrum (Figure 4.14) shows a correlation between the acetamide CH_3 signal (2.08 ppm) of the major rotamer to the δ protons (3.73 ppm) of hydroxyproline, signifying that the preferred isomer is in the *trans* conformation. There is also correlation of the minor acetamide CH_3 signal (1.93 ppm) to the minor α proton (4.52 ppm) of the prolyl ring. For simplicity's sake, we will assign only the major rotamer from here forth.

The resonances at 4.99 ppm could be readily assigned to the anomeric proton of the arabinose residue, close to the ~5.11 ppm reported by Leonard,[22] with the disparity between the numbers most likely due to the differing solvent used. The ^{13}C-^{1}H correlation spectrum (Figure 4.15, HSQC) revealed that the anomeric proton was attached to a ^{13}C resonance at 101.2 ppm, indicating the substitutents at C1 and C2 are *cis*.[110] The *J* coupling constant of the anomeric signal was 4.6 Hz which is typical for β-arabinosides (*c.f.*, *J* = 0-2 Hz for α-arabinosides). The COSY spectrum (Figure 4.16, p 144) shows a correlation of the anomeric

proton signal to a doublet of doublets at 3.96 ppm which we label H2. Resonances at 3.88 and 3.76 can be readily assigned to H3 and H4 respectively. Two signals correlate to H4, which the HSQC spectrum reveals to be attached to the same carbon at 63.9 ppm (*c.f.*, 64.1 ppm as assigned by Leonard in Art v 1), and is assigned as the diastereotopic H5 protons of the arabinose ring.

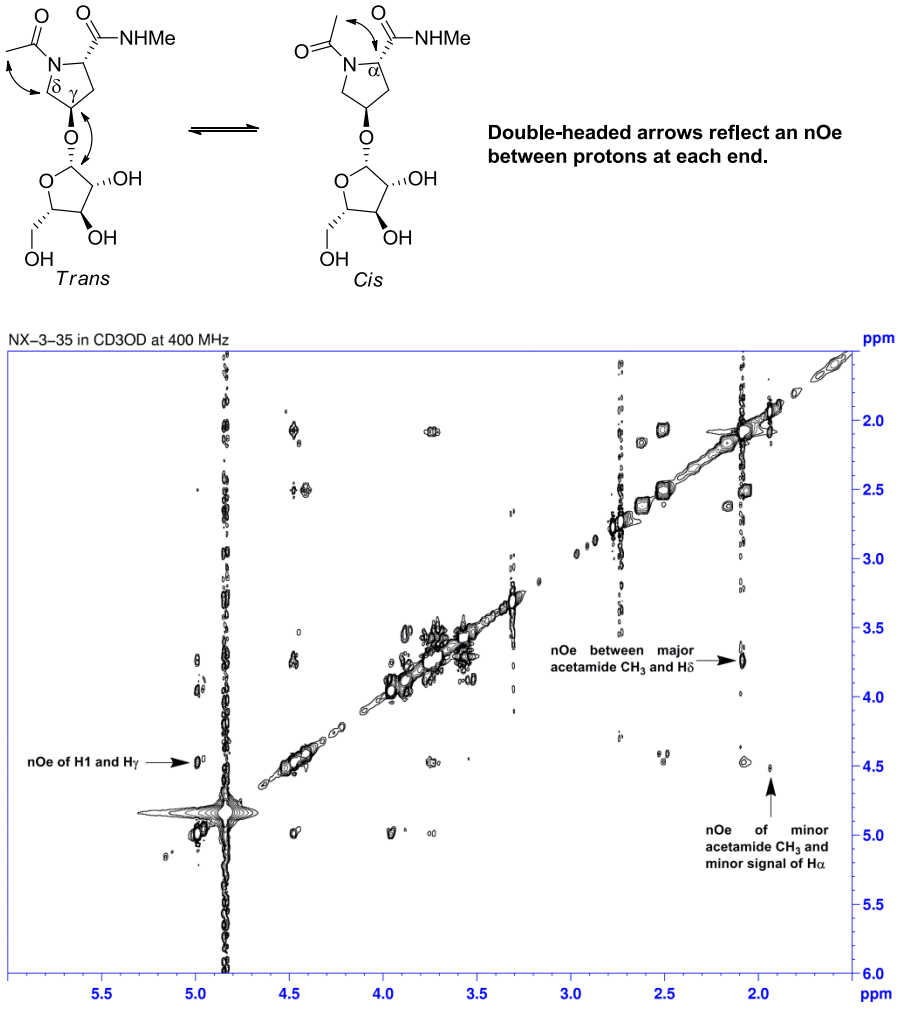


Figure 4.14 NOESY spectrum of Ac-([β-L-Ara*f*]Hyp)-NHMe

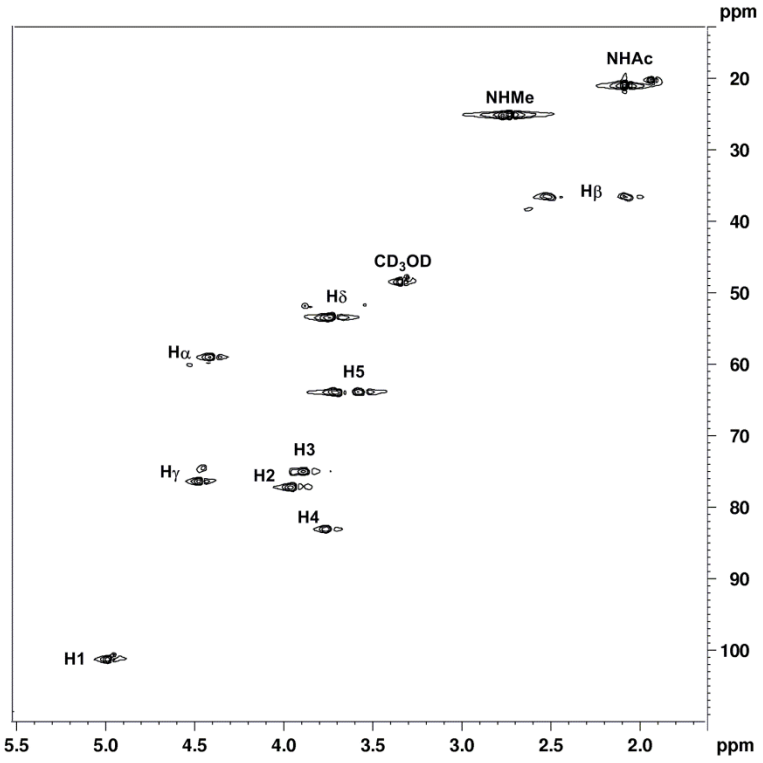


Figure 4.15 HSQC spectrum of Ac-([β-L-Ara*f*]Hyp)-NHMe

Multiplets at 2.06 and 2.50 ppm can be assigned to a pair of diastereotopic protons attached to a carbon that resonates at 36.5 ppm as seen in the HSQC spectrum. These were assigned to Hβ of the proline ring, consistent with their chemical shifts. The Hβ signals show correlations with two other signals in the COSY spectrum. The first of the two signals was assigned to Hα (4.41 ppm), as evidence by its splitting into a triplet. The second of these signals is Hγ (4.46 ppm) which was split into a multiplet as expected from its environment. The Hγ resonance showed further correlation with a pair of diastereotopic protons at 3.72 ppm which are the Hδ signals. Finally, the glycosidic linkage of hydroxyproline and arabinose was confirmed by an nOe between Hγ of Hyp (4.46 ppm) and the anomeric proton of β-Ara*f* (4.99 ppm).

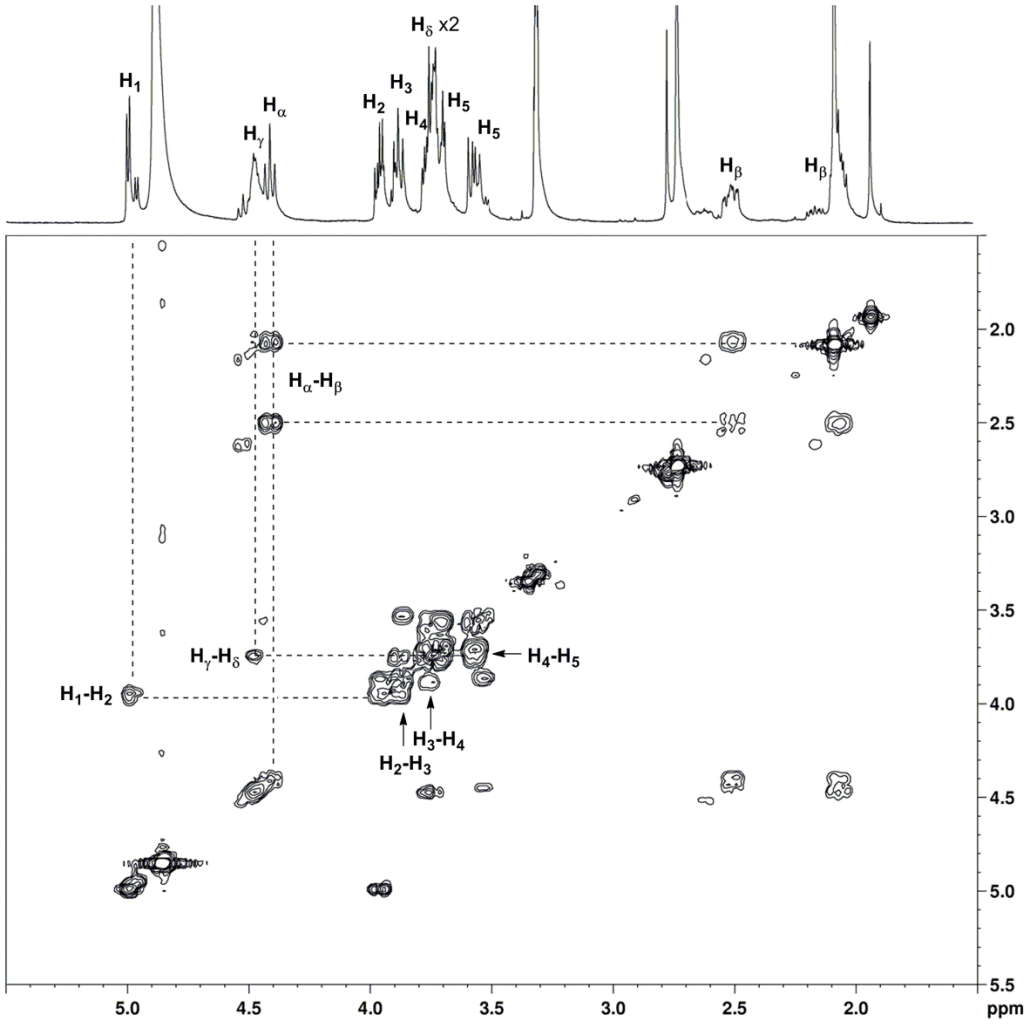


Figure 4.16 COSY spectrum of Ac-([β-L-Ara*f*]Hyp)-NHMe

Nuclear magnetic resonance spectra were acquired for the oligomers. Figure 4.17 shows a comparison of the ^{1}H NMR spectra of the four compounds. We have fully characterized the monomer and dimer using ^{1}H, ^{13}C, and 2D NMR experiments. With each additional (β-L-Ara*f*)Hyp residue added, however, the NMR spectra became increasingly more complex. The spectral assignments of trimer and tetramer were made with only 1-D NMR experiments and by extrapolation from spectral assignments of monomer and dimer.

To validate the number of residues, we compared the integration of *C*-terminal methyl amide (#H = 3 for all compounds, ~2.75 ppm) to the anomeric signal (#H = 1-4 for monomer,

dimer, trimer and tetramer, ~5.00 ppm). The *N*-terminal acetamide could also be employed for this comparison, albeit some overlap with Hβ signal made this less straightforward than using the methyl amide. Although the integration of the combined anomeric signals increased by one unit per added residue, additional anomeric peaks were not detected, signifying that all sugar residues are in a similar environment and conformation. In the larger oligomers, rotational isomeric signals were less prominent than that of the monomer. This is in contrast to the fully benzylated Boc-([β-L-Ara*f*]Hyp)$_n$-OAll peptides where each additional residue gave rise to a new pair of rotational isomeric signals, resulting in up to eight ^{13}C signals for C1 in the fully protected tetramer (**100**, Scheme 3.19). Our findings on the protected oligomers are in agreement with previous work on oligoprolines.[112] It is possible that the carbohydrate residues are contributing to the reduced number of species of these larger compounds in solution. Figure 4.17 shows the ^{1}H NMR spectra of **118**, **119**, **120**, and **1** in CD_3OD at 400 MHz (Next Page).

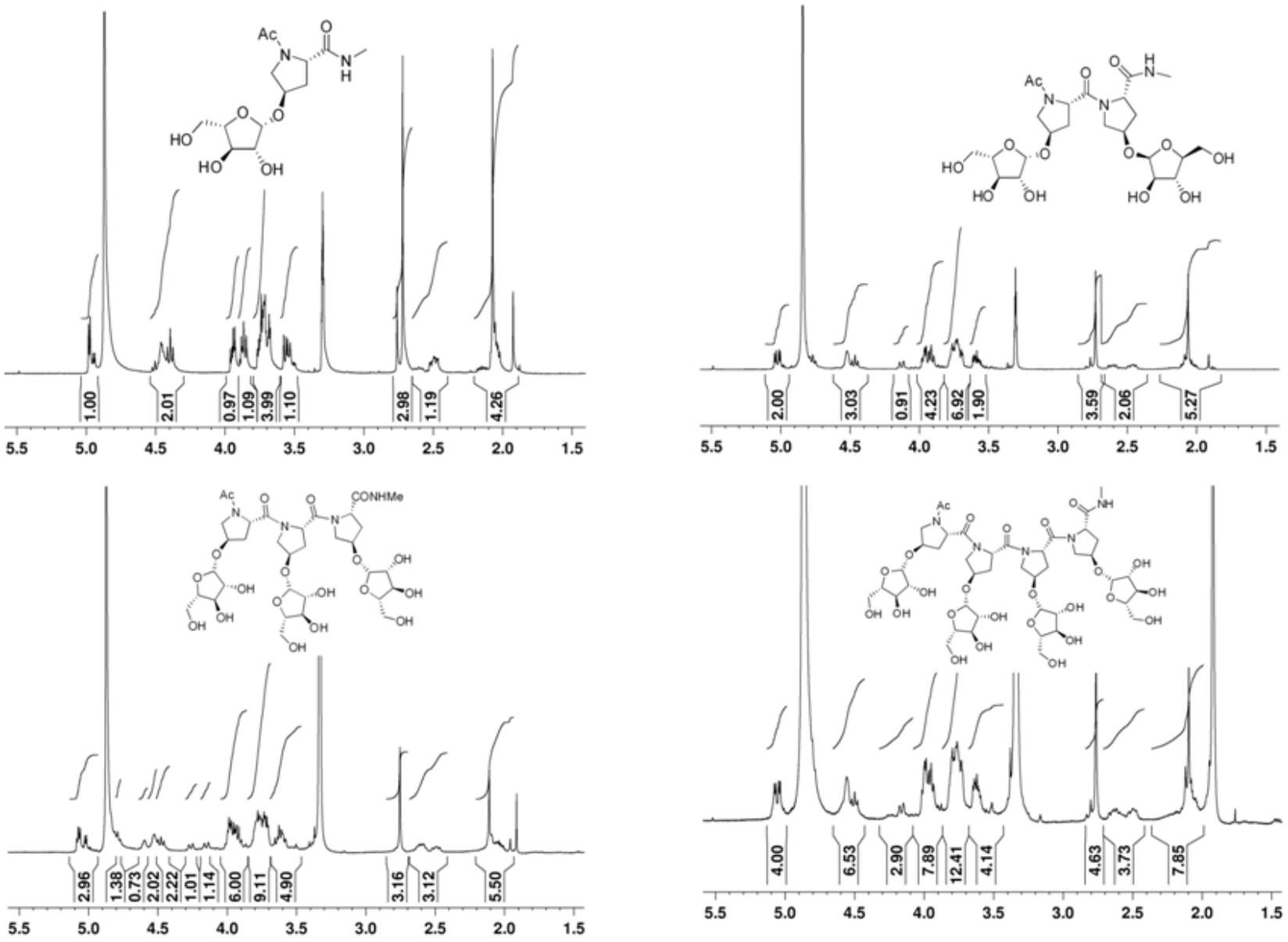


Figure 4.17 ^{1}H NMR of all four synthetic glycopeptides

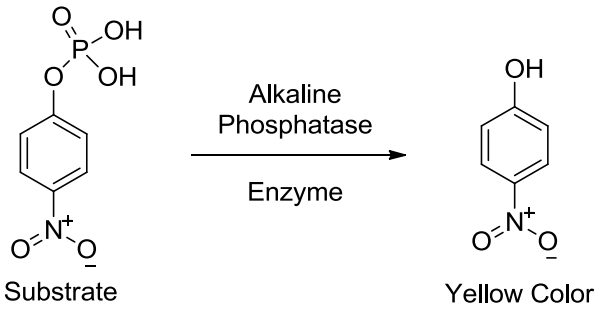
4.3 Enzyme Linked Immunosorbent Assays

In the spring of 2011, the first two synthetic glycopeptides were sent to the Altmann Group at BOKU in Vienna, Austria. Ten milligrams each of monomer **117** and dimer **118** were provided by the Taylor Group for biological testing against antibodies specific for the carbohydrate region of Art v 1. Enzyme linked immunosorbent assay (ELISA) experiments, used extensively for the detection of antibodies/antigens in serum samples, were employed to confirm the existence of glycan epitopes. The Altmann group conducted cross-inhibition ELISA experiments, also known as competitive ELISA, to determine the IgG binding properties of the synthetic glycopeptides.

First, various concentrations of monomer **117** and dimer **118** were incubated with rabbit serum containing only the anti-glycan IgGs. This serum was then added to a microtiter plate bound with natural Art v 1 to allow for possible competitive binding to the antibody. The plate was washed so that any unbound antibody was washed away. The amount of remaining antigen still bound to the surface of the plate correlates directly to the binding capacity of the synthetic glycopeptides to the antibody generated against the carbohydrate region of the natural allergen. The higher the percentage of antigen still present in the wells signifies a higher percentage of the synthetic glycopeptides binding to the antibody.

In order to measure this displacement, the remaining bound anti-glycan IgGs were treated with an alkaline phosphatase-conjugated goat anti-rabbit IgG that is specific for the bound antigen. The plate was washed again to remove any unbound antibody-enzyme conjugate. Upon activation of the enzyme with 0.1% p-nitrophenyl phosphate (Scheme 4.1), a chromogenic substrate, and a 0.1 M diethanolamine (pH 9.8) buffer, the plate was read immediately at 405/620 nm with an SLT-spectra plate reader to obtain quantitative results on

the amount of antigen remaining. Figure 4.18 shows the initial results of the ELISA inhibition experiment with the synthetic glycopeptides at millimolar concentration.



Scheme 4.1 Activation of ALP with *p*-nitrophenyl phosphate

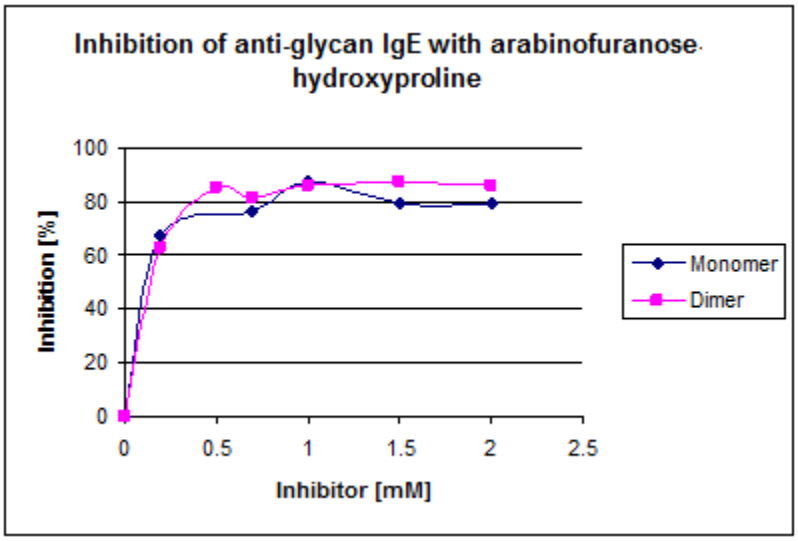
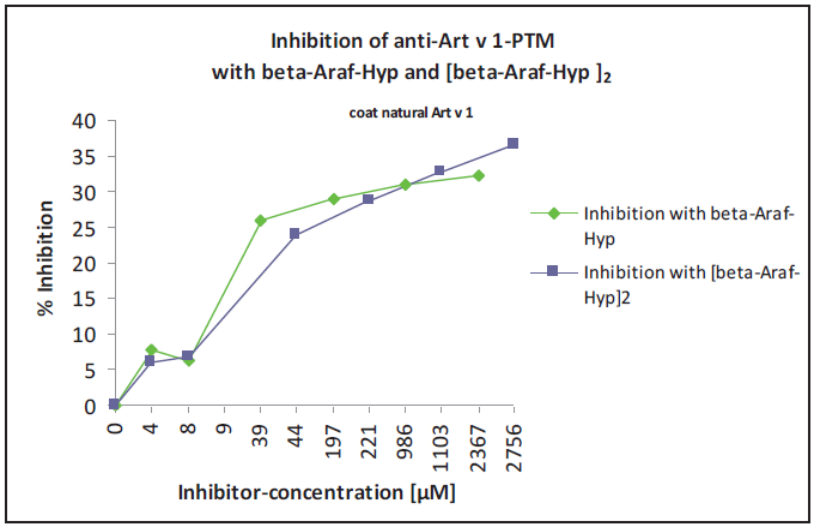


Figure 4.18 ELISA inhibition experiments of synthetic glycopeptides

At the highest of concentrations (2 mM), the % inhibition for both the monomer and dimer were similar to one another, reaching 80% at ~0.5 mM; these initial results were encouraging. Unfortunately, subsequent repeated assays with diluted samples showed inconsistencies (Figure 4.19). The inhibition potency of monomer and dimer varied in the experiments for unexplained reasons. However, in all three experiments presented here, monomer and dimer inhibit Art v 1 binding to a similar extent. The results reported herein are unpublished work from the Altmann Group.[113]



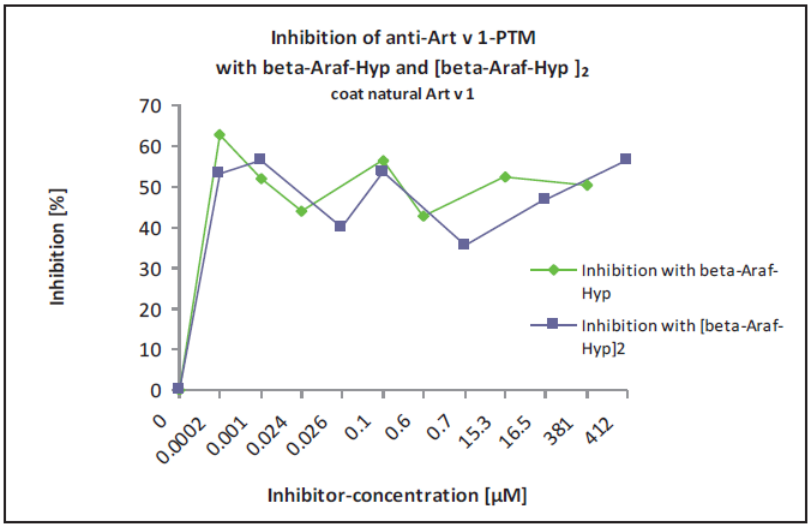
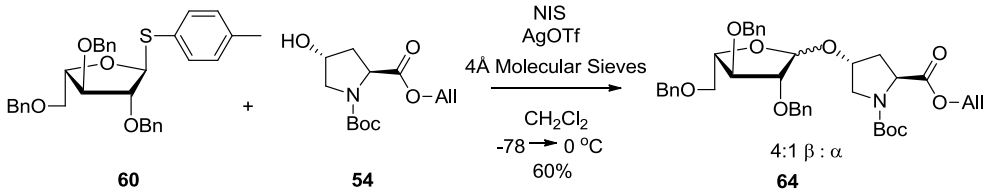


Figure 4.19 Varied concentrations of synthetic glycopeptides in inhibition experiments

4.4 Summary

It was the goal of this dissertation to find the minimal carbohydrate epitope of the Art v 1 allergen. A key intermediate in the assembly of relevant oligomers was a β-arabinoside of hydroxyproline, a 1,2-*cis* glycoside that is a challenge to synthetic organic chemistry. Towards the synthesis of this intermediate, we prepared *N*-*tert*-butoxycarbonyl-trans-4-hydroxy-L-proline allyl ester (**54**) for glycosylation with p-cresyl 2-O-benzyl-3,5-O-(di-tert-butylsilylene)-1-thio-α-L-arabinofuranoside (**42**). Unfortunately, use of this conformationally restricted bicyclic donor **60**

resulted in low yields and complex mixtures. Instead, we employed *p*-cresyl 2,3,5-*O*-benzyl-1-thio-α-L-arabinofuranoside (**60**), for glycosylation of hydroxyproline. Using careful temperature control, we were able obtain the Ara-Hyp glycoside **64** with 4:1 β:α ratio in 60% yield. Flash column chromatography could be used to separate the anomers.



Scheme 4.2 Glycosylation of Boc-Hyp-OAll by sulfide donor

In preparing the oligomers of hydroxyproline arabinosides, we unmasked the *N*- and *C*-termini of Boc-([β-L-Ara*f*]Hyp)-OAll in independent experiments. Coupling of the free acid and amine with HATU gave the Boc-([β-L-Ara*f*]Hyp)$_2$-OAll dimer (**85**) in 60% yield. The trimer **99** could be assembled by the coupling of Boc-([β-L-Ara*f*]Hyp)-OH with H-([β-L-Ara*f*]Hyp)$_2$-OAll [1+2] or Boc-([β-L-Ara*f*]Hyp)$_2$-OH with H-([β-L-Ara*f*]Hyp)-OAll [2+1], with each strategy affording chemical yields of ~35%. Coupling of a Boc-([β-L-Ara*f*]Hyp)$_2$-OH and H-([β-L-Ara*f*]Hyp)$_2$-OAll gave the tetramer **100** in 49%.

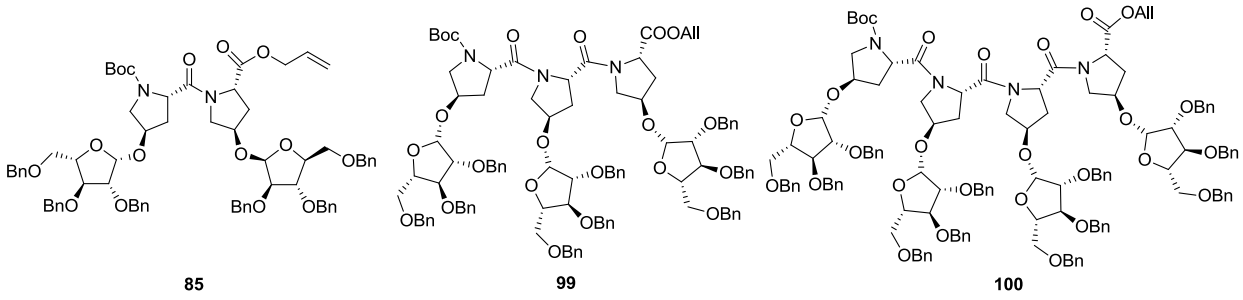
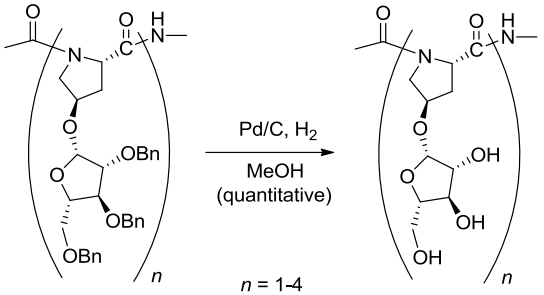


Figure 4.20 Protected oligomers

Installation of amide end-caps on Boc-([β-L-Ara*f*]Hyp)-OAll gave Ac-([β-L-Ara*f*]Hyp)-NHMe in 42% yield over 4 steps. Switching to a more convergent and higher yielding route, we chose to utilize pre-endcapped glycosidic building blocks for the preparation of end-capped oligomers. Position-specific glycosidic building blocks Ac-([β-L-Ara*f*]Hyp)-OMe (*N*-terminal) and

Boc-([β-L-Ara*f*]Hyp)-NHMe (*C*-terminal) were prepared. With this strategy, fragment condensation of end-capped building blocks afforded dimer **104**, trimer **114**, and tetramer **117** in 48%, 35%, and 15% respectively. Global debenzylation of the end-capped glycopeptides gave the four deprotected compounds in quantitative yield.



102: *n* = 1
104: *n* = 2
114: *n* = 3
117: *n* = 4

118: *n* = 1
119: *n* = 2
120: *n* = 3
1 : *n* = 4

Scheme 4.3 Global debenzylation

Circular dichroism data were obtained for the synthetic glycopeptides. The monomer spectrum showed it to be unordered as expected. Analysis of the CD spectra showed that the glycosylated proline oligomers exhibited characteristic polyproline II helical conformation. However, to our surprise, the dimer displayed order in its elliptical curve (λ_{max} = 220 nm, λ_{min} = 199 nm). The trimer (λ_{max} = 222 nm, λ_{min} = 203 nm) and tetramer (λ_{max} = 220 nm, λ_{min} = 200 nm) both exhibited significant PPII helical conformation.

Nuclear magnetic resonance spectroscopy was used to characterize all target glycopeptides. ^{1}H, ^{13}C, and various 2-D NMR were used to identify key resonances in the comparison of the synthetic glycopeptides with glycoprotein isolated from the natural allergen.

In closing, the synthesis and oligomers described herein enable the further study of this important class of compounds. Our homogeneous compounds, characterized with the rigor of organic chemistry, lay the foundation for unambiguous biological studies that were not possible

with the trace amount of heterogeneous material available from degradation of the native Art v 1 protein.

4.5 Future Work

The obvious next step in the project is to conduct ELISA experiments on trimer and tetramer in varying concentrations. With the completion of the CD experiments, trimer and tetramer are ready to be sent to Vienna. Despite reproducibility issues, the fact that monomer and dimer have behaved similarly may suggest that the large oligomers will as well. The direction of the project thereafter will be conditional upon those results. In any event, we propose herein a plan for the continuation of the investigation of the carbohydrate epitope of Art v 1.

4.5.1 Incorporation of the β-Ara-Hyp motif into longer pepitides

While we have synthesized the tetraproline component of the tail section of Art v 1, we do not yet know whether or not this is optimal, *vis-à-vis* biological response. While the monomer and dimer seemed to be at least partially effective, it is possible that increasing the length of the peptide chain may be beneficial to the activity of the glycopeptides. Table 4.3 shows the amino acid sequence of the carbohydrate domain of Art v 1. Note that the proline residues typically follow a serine or alanine residue. In fact, the amino acid sequence SPP is found thrice in this sequence and SPPPP twice. The sequences APP and APPP are also found. It is interesting as to whether or not an extended glycopeptide sequence, synthesized to include these serine and alanine residues, and perhaps more than one glycocluster, would have an effect on antibody binding. If so, will an even longer sequence of the tail section of Art v 1 be worth investigating?

Table 4.3 Amino acid sequence in the polyproline domain

56-60	61-70	71-80	81-90	91-100	100-108
S**PP**GA	T**P**A**PP**GAA**PP**	**P**AAGGS**P**S**PP**	ADGGS**PPP**A	DGGS**PP**VDGG	S**PPPP**STH

4.5.2 Antibody generation

Glycosylated proteins have been known to have antigenic properties.[114] Advances in carbohydrate research have further illuminated their potential as vaccines.[115] However, the low immunogenicity of carbohydrate antigens continues to prove problematic. Polysaccharides are T-cell independent, meaning they do not induce immunological memory. To this end, carbohydrate antigens have been coupled to carrier proteins to heighten their immunogenicity.[116]

Avery and Goebel first introduced the technique to enhance the immunogenicity of polysaccharide antigens.[117] More recently, this hapten-carrier protein conjugate strategy has been regularly employed for bacterial carbohydrates.[118] The covalent linkage of the polysaccharide and the carrier protein has been achieved by various techniques (*i.e.*, carbodiimide coupling, reductive amination, etc).[119]

Covalent linkages to our synthetic glycopeptides can be made by *N*-terminal conjugation. The free amine will be modified for attachment via a spacer unit bearing a carboxylic acid which permits conjugation (Figure 4.21). The glycoprotein conjugate will then be used to raise a rabbit antiserum specific against the homogeneous, synthetic β-Ara-Hyp epitope. Monoclonal antibodies generated using the glycoprotein conjugate can potentially be useful in screening for cross-reactivity in other plant allergens. Finally, the glycoprotein conjugates can be used to determine binding affinities for rabbit and human IgG and IgE.

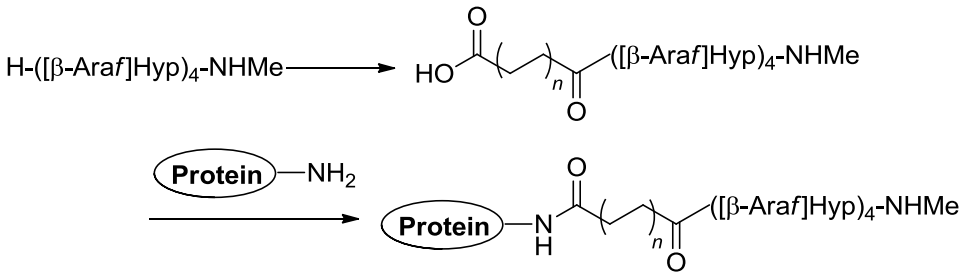
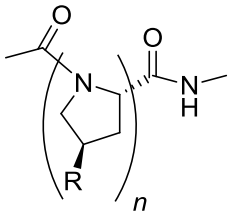


Figure 4.21 Proposed conjugation of synthetic glycopeptides to carrier proteins

4.5.3 Effect of glycosylation on oligoproline conformation

The Taylor group has made fundamental contributions to conformational determinates in proline-containing peptides.[57, 120] This dissertation opens the door to studies on consecutive proline residues as well as modified proline oligomers. Molecular dynamics studies have shown the predominant conformation of end-capped oligoprolines to be *trans*,[121] which indicates a PPII helix. Utilizing NMR and circular dichroism spectroscopy, investigation of the twelve compounds in Table 4.4 would allow us to see the changes in their conformation with regards to number of proline residues and degree of post-translational modification. This would give greater context to the results described in Section 4.14.

Table 4.4 Target oligoproline compounds



	124	**125**	**126**	**127**	**128**	**129**	**130**	**131**	**118**	**119**	**120**	**1**
n	1	2	3	4	1	2	3	4	1	2	3	4
R	H				OH				*O*-β-L-Ara*f*			

4.5.4 Synthetic glycopeptides as diagnostic tools

The development of modern diagnostic tools has been paramount in the identification of allergies.[122] Two of the most commonly used screening methods are the skin prick test (SPT) and allergy blood tests. In order to pinpoint the exact source of the allergen, investigations have focused on identifying cross-reactive pollens of the Asteraceae family.[123] Pollen from ragweed, chrysanthemum, and dandelion may share similar epitopes (β-Ara-Hyp) with mugwort pollen. Further related plants such as feverfew and sunflower may also contain the β-Ara-Hyp epitope as these species all produce a homolog of Art v 1. Most notably, the allergen of ragweed, Amb a 4, has 50% homology to Art v 1 and was found to contain small amounts of β-Ara-Hyp.[31] Furthermore, natural Art v 1 was found to inhibit IgE binding of Amb a 4. In order to evaluate

the relevance of the β-Ara-Hyp epitope, we propose that a specific anti-β-Ara-Hyp serum be generated in order to screen a wide array of plant pollens.

4.6 Experimental Section

4.6.1 Circular dichroism spectroscopy

Sample compounds were lyophilized for 24 h prior to dilution to a concentration of 0.4 mM with water. The pH of the samples was determined at rt and found to be 6.80, 7.38, 8.79, and 9.48 for compounds **118**, **119**, **120**, and **1** respectively. Circular dichroism measurements were carried out using a JASCO J-815 spectrometer. For analysis, 175 µL of the sample was loaded into a quartz cell with a path length of 0.1 cm. The CD spectra were recorded at a scan rate of 20 nm per min, data pitch of 1.0 nm, and bandwith of 2.0 nm. The accumulation of three scans was averaged for each sample, after which a blank of the solvent was subtracted. The CD signal was converted to molar ellipticity per mean residue ([θ]) and the data was smoothed by application of the Savitzky-Golay algorithm.

4.6.2 NMR spectroscopy

NMR spectra were obtained using a Bruker AV-400 or Varian 700 MHz spectrometer. Proton NMR data is reported in ppm downfield from TMS as an internal standard. Disodium 3-trimethylsilyl-1-propane-sulfonate (DSS) was used to reference ^{1}H NMR spectra run in D_2O. Minor rotational isomers are reported in parentheses when significant amount exist.

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APPENDIX: LETTERS OF PERMISSION

Figure 1.6 – Page 17

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Figure 1.11 – Page 13

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Figure 2.5 – Page 26

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Figure 4.4 – Page 131; Figure 4.5 – Page 132

Title:	Host−Guest Study of Left-Handed Polyproline II Helix Formation†
Author:	Melissa A. Kelly et al.
Publication:	Biochemistry
Publisher:	American Chemical Society
Date:	Dec 1, 2001

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Figure 4.8 - Page 134

Title:	Critical chain length for polyproline-II structure formation in H-Gly-(Pro)n-OH
Author:	N. HELBECQUE,M.H. LOUCHEUX-LEFEBVRE
Publication:	Chemical Biology & Drug Design
Publisher:	John Wiley and Sons
Date:	Jan 12, 2009

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Figure 4.9 – Page 135

Title:	Contiguous O-Galactosylation of 4(R)-Hydroxy-l-proline Residues Forms Very Stable Polyproline II Helices
Author:	Neil W. Owens, Jörg Stetefeld, Erika Lattová, and Frank Schweizer
Publication:	Journal of the American Chemical Society
Publisher:	American Chemical Society
Date:	Apr 1, 2010

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VITA

Ning Xie was born in Yangzhou, China, where he lived and attended school up until the 3rd grade. Two months before his 8th birthday, Ning came to Baton Rouge, Louisiana, to join his father, who was currently a doctoral candidate at Louisiana State University under Professor Milton C. Rush, and mother. He would live in Baton Rouge for the better part of his life, along with a short stint in Crowley, Louisiana.

Ning Xie received his Bachelor of Science degree in Chemistry in 2007 from LSU. He performed organic chemistry experiments as an undergraduate in the laboratory of Professor Robert Hammer where he first conducted peptide chemistry. In the fall of 2007, Ning was accepted into the doctoral program in the chemistry department at LSU where he is currently a doctoral candidate in organic chemistry working under the tutelage of Professor Carol M. Taylor. His graduate dissertation work involved the synthesis of oligomers of arabinosylated hydroxyproline and the investigation of their allergenic potential. Ning is a member of the American Chemical Society.